Protecting the Ischemic Heart

Pharmacological Protection Against Myocardial Ischemia in Rats In Vivo and In Vitro

Inauguraldissertation

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Summary

Today in Switzerland approximately 40% of all deaths are due to cardiovascular diseases. More than half of these are due to ischemic heart disease. Among the fatal consequences of ischemic heart disease are life-threatening arrhythmias and myocardial infarction. This thesis contains two studies focusing on pharmacological protection against the consequences of myocardial ischemia. The first study focuses on reperfusion arrhythmias and the second study focuses on reducing myocardial infarction size.

The first study is based on the finding, that the angiotensin II receptor type 1 (AT_1) blocker losartan might be associated with a lower mortality than that found with the angiotensin converting enzyme (ACE) inhibitor captopril in elderly heart failure patients. This difference appeared to arise largely from a decrease of sudden death in losartan-treated patients. As ventricular tachyarrhythmias, particularly ventricular fibrillation (VF), contribute largely to sudden death, losartan has been suggested to be antiarrhytmic. Therefore, the goal of the present study was to assess acute antiarrhythmic effects of losartan and enalaprilat in hypertrophied rat hearts during low-flow ischemia/reperfusion. In dose-finding experiments in non-hypertrophied isolated perfused hearts, we performed dose-response curves of losartan (1 nM to 1 mM) and enalaprilat (1 nM to 1 mM) studying monophasic action potential duration at 90% repolarisation ($MAPD_{90\%}$) and ventricular fibrillation (VF) threshold. Subsequently, we determined the effects of losartan and enalaprilat in therapeutically relevant concentrations on ventricular tachyarrhythmias induced by low-flow ischemia/reperfusion in hearts demonstrating left ventricular (LV) hypertrophy 70 days after aortic banding. We found that neither drug significantly affected $MAPD_{90\%}$ or VF threshold in non-hypertrophied hearts. Similarly in hypertrophied hearts, neither drug significantly affected the incidence or the duration of ventricular tachyarrhythmias (ventricular tachycardia and VF) during low-flow ischemia. However, 1 μM losartan significantly reduced the duration of ventricular tachyarrhythmias during reperfusion. In conclusion, neither losartan nor enalaprilat is acutely antiarrhythmic in hypertrophied rat hearts during low-flow ischemia. During reperfusion, however, losartan but not enalaprilat exerts acute antiarrhythmic effects.

The second study focuses on nuclear factor kappa-B (NF-κB), an ubiquitous transcription factor, that is involved in immune, inflammatory, and stress responses. Accordingly NF-κB might play an important role in myocardial ischemia-reperfusion injury. Dimethyl fumarate (DMF), an orally available small molecule drug for psoriasis patients, has been shown to inhibit TNFα-induced nuclear entry of NF-κB in endothelial cells in vitro. Therefore we postulated that DMF might also affect NF-κB in cardiomyocytes in vivo and thus reduce myocardial infarction following ischemia and reperfusion. Male Sprague-Dawley rats undergoing left coronary artery occlusion for 45 min received either DMF (10 mg/kg b.w.) or vehicle 90 min before ischemia as well as immediately before ischemia. The positive control group received the vehicle plus ischemic preconditioning (2 x 5 min ischemia, each followed by 5 min of reperfusion). After 120 min of reperfusion, the hearts were stained with phthalocyanine blue dye (area at risk) and 2,3,5-triphenyltetrazolium chloride (infarct area). Heart rate and QT_C interval were determined on the recorded ECG. Additionally, acute hemodynamic and electrophysiologic effects of DMF were determined in dose-response curves in isolated perfused rat hearts. We found that myocardial infarct size was significantly smaller in rats that had received DMF or ischemic preconditioning than in control rats. Neither heart rate nor QT_C interval differed between DMF-treated and untreated animals. Dose-response experiments (0.1 μM to 1000 μM) in isolated perfused rat hearts excluded acute hemodynamic (coronary flow and left ventricular pressure) or electrophysiologic effects (action potential duration) as a mechanism for the antinecrotic effects of DMF. In conclusion, this study in rats in vivo demonstrates that DMF reduces myocardial infarct size after ischemia and reperfusion. The molecular mechanisms for this effect of DMF on myocardial infarct size are presently not clear.

Abbreviations

ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotropic hormone
AT_1	Angiotensin II receptor type 1
AT_2	Angiotensin II receptor type 2
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
BK receptor	Bradykinin receptor
bpm	Beats per minute
b. w.	Body weight
DMF	Dimethyl fumarate
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
ECG	Electrocardiogram
ELITE	Evaluation of Losartan in the Elderly Study
i-□□	Inhibitor □□
I_{kr}	Outward rectifying potassium current (rapid)
I_{ks}	Outward rectifying potassium current (slow)
iNOS	Inducible nitric oxide synthase
I_{to}	Transient outward current
i. v.	Intravenous
K_{ATP}	ATP-sensitive potassium channel
LAD	Left anterior descending artery
LV	Left ventricular
LVDP	Left ventricular developed pressure
MAPD $_{90\%}$	Monophasic action potentials at 90% repolarisation
MHF	Methyl hydrogen fumarate
mRNA	□ essenger ribonucleic acid
NF-□□	Nuclear factor □□
NO	Nitric oxide

QTc	Corrected QT-Interval
RAAS	Renin-angiotensin-aldosteron-system
SD	Standard deviation
TNF-□	Tumor necrosis factor-□
TTC	2,3,5-triphenyltetrazolium chloride
VF	Ventricular fibrillation
VFT	Ventricular fibrillation threshold
VT	Ventricular tachycardia

1 Introduction

Cardiovascular diseases are the major cause of death in Switzerland today. Approximately 40% of all deaths are due to cardiovascular heart disease. Among the fatal consequences of ischemic heart disease are life-threatening arrhythmias and myocardial infarction (Burckhardt et al 2003). Extensive research over the last decades has led to a better understanding of the pathophysiology of injuries associated with ischemia-reperfusion. Futhermore, understanding these mechanisms allows the development of new strategies to treat these life-threatening consequences. This thesis contains two studies focusing on this topic.

1.1 Ischemia and myocardial infarction

Life-threatening arrhythmias can be a consequence of myocardial ischemia. This myocardial ischemia is characterized by an imbalance between myocardial oxygen supply and demand (Fig. 1). In some situations this imbalance is caused by a reduction of blood flow and oxygen supply secondary to increased coronary vascular tone, intracoronary platelet aggregation, or thrombus formation. This condition, termed supply ischemia, is responsible for myocardial infarction and most episodes of unstable angina (Ganz & Ganz 2001).

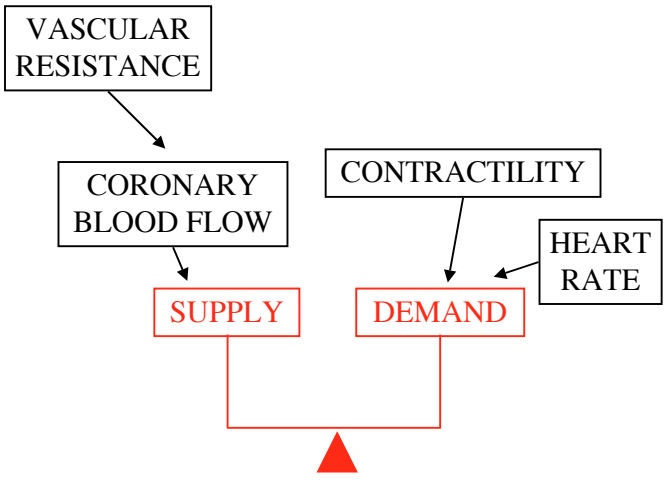


Figure 1: Factors influencing myocardial oxygen supply and demand. Modified from (Ganz & Ganz 2001).

Low-flow ischemia is characterized not only by oxygen deprivation but also by inadequate removal of metabolites due to reduced perfusion and by loss of vascular turgor. Buildup of tissue metabolites, especially inorganic phosphate, reduces calcium sensitivity of myofilaments, thereby diminishing contractility (Ganz & Ganz 2001).

The working heart has a sustained energy demand. This demand can be met only by the efficient pathways of oxidative phosphorylation in mitochondrias, which requires that the coronary circulation deliver an uninterrupted supply of substrates, notably oxygen. These energy demands are so big that the entire ATP content of the heart turns over every 4 to 5 seconds. For this reason, the heart cannot tolerate ischemia. This explains why coronary artery occlusion is followed almost immediately by loss of function, and within hours, by cell death (Antman & Braunwald 2001; Katz 2001)(Fig. 2).

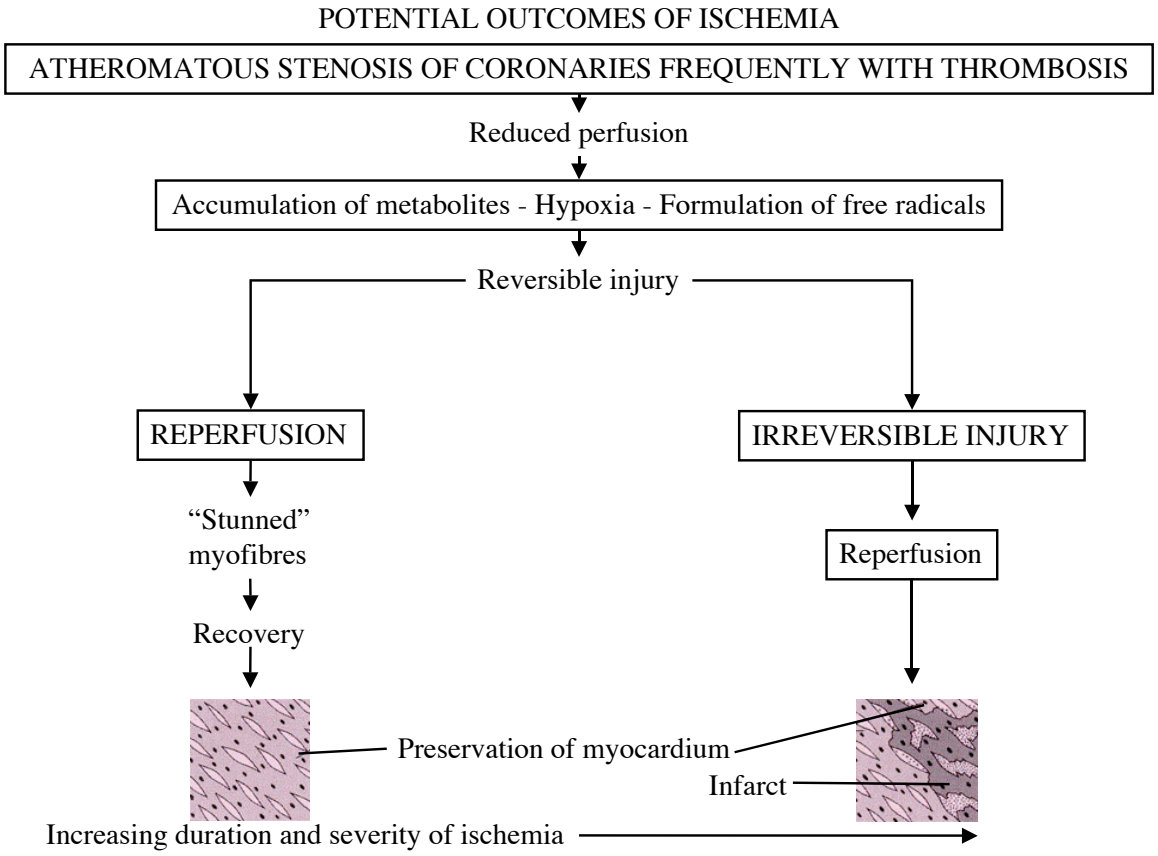


Figure 2: Several potential outcomes of reversible and irreversible ischemic injury to the myocardium. Modified from (Antman & Braunwald 2001).

If a heart suddenly becomes severely damaged, as during myocardial infarction, the pumping ability of the heart is immediately depressed. This results in a reduced cardiac output and in a damming of blood in the veins, resulting in increased venous pressure (Guyton & Hall 2000).

The process of reperfusion, although beneficial in terms of myocardial salvage, may come at a cost, due to a process known as reperfusion injury. This consists of lethal reperfusion injury (reperfusion-induced death of cells), vascular reperfusion injury (progressive damage to the microvasculature), stunning (salvaged myocytes display a prolonged period of contractile dysfunction after restoration of blood flow owing to abnormalities of intracellular biochemistry leading to reduced energy production), and reperfusion arrhythmias (bursts of ventricular tachycardia and, on occasion, ventricular fibrillation that occurs within seconds of reperfusion) (Ganz & Ganz 2001).

In 1986 a phenomenon termed ischemic preconditioning was discovered (Murry et al 1986). Dog hearts in vivo were subjected to four 5-min coronary occlusions, separated by 5 min reperfusion, before a sustained 40 min ischemic insult. The investigators found these preceding brief periods of ischemia and reperfusion were protective, reducing infarct size after subsequent prolonged ischemia to 25% of that seen in the control group.

If myocardial infarction is not lethal, a remodeling process starts which leads to hypertrophy. This takes place because the adult heart cannot adapt the cell loss by generating new cells, but cardiac myocytes do become larger (hypertrophy). This hypertrophic response is well suited for the heart, which must beat continuously and so cannot suspend its contractile activity to generate new myocytes (Katz 2001).

1.2 Hypertrophy, heart failure, and the renin-angiotensin-aldosterone-system

Cardiac hypertrophy is a process wherein there is an increase in chamber mass produced largely by an increase in the size of cardiomyocytes. Pathologic hypertrophy is an

important adaptive response to abnormal increase in cardiac work. Initially, the increase in cardiac mass serves to normalize wall stress and permit normal cardiovascular function (Walsh & Dorn 1998). If the compensatory response is adequate to "match" the work demands, a period of relative stability ensues. However, if the extent or form of myocardial remodeling is insufficient, or if the magnitude of the overload increases further, there is additional deterioration in myocardial function. This is a consequence of "afterload mismatch," that is, inadequate hypertrophy to normalize mechanical stress on the myocyte; and a vicious cycle is created (Fig. 3) (Colucci & Braunwald 2001).

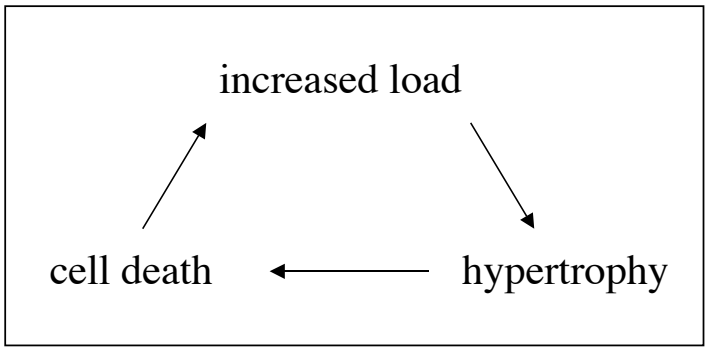


Figure 3: Vicious cycle in which overload causes the heart to hypertrophy, which causes cell death, which increases the overload, etc. (Katz 2001).

Pathologic hypertrophy may be caused by pressure overloading such as hypertension or aortic coarctation (Walsh & Dorn 1998). Cardiac enlargement is the first compensatory mechanism recognized in clinical heart failure. The heart, like any pump, moves fluid from a region of low pressure (the veins) to one at higher pressure (the arteries). Therefore, heart failure can reduce the forward flow of blood into the aorta and pulmonary artery, cause blood to back up behind the heart, or both (Katz 2001). This leads to an inability to maintain a normal blood pressure and organ perfusion. Hypotension evokes baroreflexes to increase sympathetic adrenergic discharge, thereby stimulating β_1 renal receptors involved in renin release. Together with stimulation of chemoreflexes as well as with the decreased renal perfusion, which also enhances renin release, this mechanism is responsible for the enhanced activity of the renin-angiotensin-aldosterone-system (Fig. 4) in heart failure patients (Opie & Gersh 2001). This leads to increased levels of angiotensin II and aldosterone.

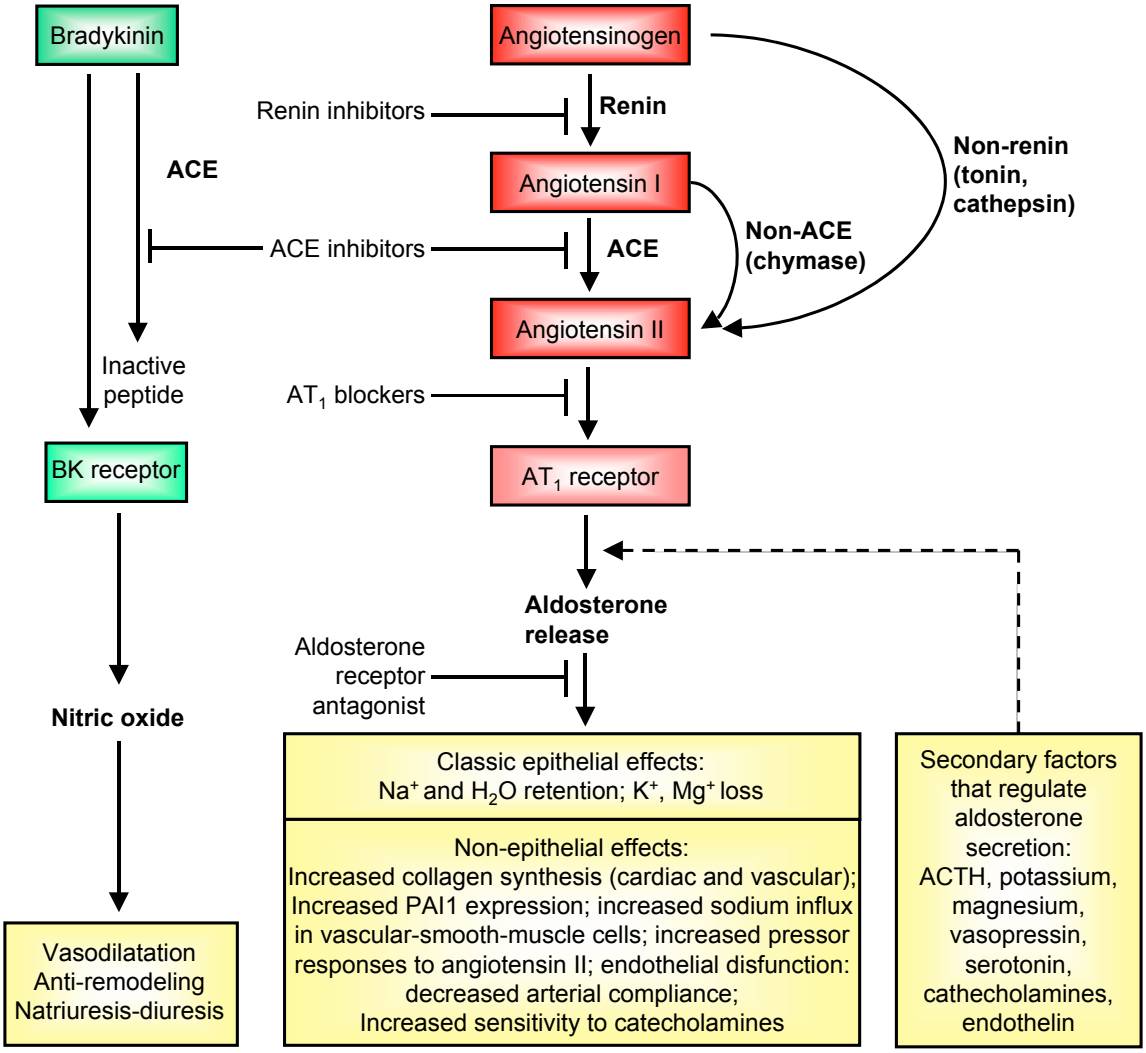


Figure 4: Renin - a protease that is secreted into the circulation in response to various physiological stimuli - cleaves the protein angiotensinogen to produce the inactive decapeptide angiotensin I. Cleavage of angiotensin I by angiotensin-converting enzyme (ACE) produces the active octapeptide angiotensin II (as shown, ACE also inactivates bradykinin, and there are alternative routes for the generation of angiotensin II). Angiotensin II activates the angiotensin II type 1 (AT1) receptor - a member of the G-protein-coupled-receptor superfamily - which has various effects. Many of these effects, such as vasoconstriction and stimulation of aldosterone synthesis and release (which leads to sodium retention), tend to elevate blood pressure. Only the aldosterone-mediated effects are shown here. Angiotensin II also activates the AT2 receptor, the effects of which are less well understood, but which could antagonize many of the effects of activation of the AT1 receptor. Various points shown in the renin-angiotensin-aldosterone cascade have been the targets for pharmacological intervention, and inhibitors of ACE, angiotensin-receptor blockers and aldosterone-receptor antagonists are in clinical use at present. ACTH, adrenocorticotropic hormone; BK receptor, bradykinin receptor (Zaman et al 2002).

Angiotensin II is a potent peripheral vasoconstrictor and contributes, along with increased adrenergic activity, to the excessive elevation of systemic vascular resistance and the vicious cycle already referred to in patients with heart failure. Angiotensin II also enhances the adrenergic nervous system's release of norepinephrine. Activation of the AT_1 receptor by angiotensin II leads to an increase in aldosterone release. Aldosterone has potent sodium-retaining properties and contributes to the development of edema. Therefore, it is not surprising that interruption of the renin-angiotensin-aldosterone axis by means of an ACE inhibitor, or an AT_1 blocker, reduces system vascular resistance, diminishes afterload, and thereby elevates cardiac output in heart failure (Colucci & Braunwald 2001). The adverse effects of renin-angiotensin activation in hypertension and in heart failure are mediated via the stimulation by angiotensin II of the receptor subtype AT_1, which the angiotensin receptor blockers, such as losartan, specifically block. This is potentially a more efficient and safer mechanism than inhibition of ACE in reducing the effects of angiotensin II, especially bearing in mind the potential synthesis of angiotensin II by non-ACE-dependent paths catalyzed by chymase (Opie & Gersh 2001). ACE inhibitors interfere with the degradation of bradykinin to inactive peptides, as angiotensin II receptor blockers do not. One appealing, but not conclusively shown mechanism of antihypertensive effects of ACE inhibitors is potentiation of bradykinin, with resultant stimulation of nitric oxide synthesis and release. Through stimulation of the B2-receptor subtype bradykinin stimulates release of vasodilator prostaglandins, and of nitric oxide, with resultant antioxidant, antihypertensive, and vasoprotective effects. The importance of bradykinin-mediated effects in patients treated with ACE inhibitors remains to be determined (Zaman et al 2002).

Cardiac enlargement, as hypertrophy and heart failure, which prolongs and disorganizes the spread of the wave of depolarization, is an important risk factor for cardiac arrhythmias.

1.3 Arrhythmias

The mechanisms responsible for cardiac arrhythmias are generally divided into categories of disorders of impulse formation, disorders of impulse conduction, or combinations of both (Rubart & Zipes 2001). Arrhythmias may result in insufficient blood flow to the organs, which my cause dizziness, inadequate function of important organs, stroke or even death. Arrhythmias can occur in the atria and in the ventricle. This thesis focuses on ventricular arrhythmias only.

There are two general types of arrhythmia: bradycardias, where the heart beats too slowly, and tachycardias, where the heart rate is too rapid. Each type includes many specific arrhythmias. The most common causes responsible for bradyarrhythmias are slowed pacemaker activity (chronotropy) and depressed action potential conduction (dromotropy). The former is caused by changes in the ionic currents responsible for pacemaker activity in the sinus node, and the latter, often called block, occurs when conduction of this impulse to the ventricles is impaired. Most tachyarrhythmias are described in terms of their clinical features because it is difficult and often impossible to define the pathophysiology of a given arrhythmia. A single early beat is usually called a premature systole, and a series of at least four premature systoles is a tachycardia. A complete disorganization of depolarization, where there is no effective beating, is fibrillation (Walker et al 1988; Katz 2001). The mechanisms that account for most of the tachyarrhythmias are accelerated pacemaker activity, triggered depolarization, and reentry. Accelerated firing of pacemaker cells in the sinus node causes sinus tachycardia, whereas early and late afterdepolarizations can cause premature systoles and sustained tachyarrhythmias in the atria, His-Purkinje system, and ventricles. Afterdepolarizations are spontaneous depolarizations that appear during and after repolarization (phase 3, Fig. 5) especially when the heart becomes calcium overloaded. Large afterdepolarizations can generate propagated action potentials, which are important causes of lethal arrhythmias. The term reentry stands for a reentrant beat that is caused when a single impulse traveling through the heart gives rise to two or more responses (Katz 2001).

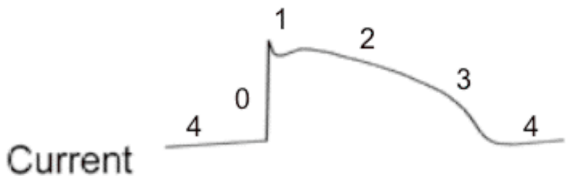


Figure 5: Phases of a purkinje fiber action potential in human beings: Phase 0 (upstroke) corresponds to depolarization, and phase 3 (repolarization) to repolarization in skeletal muscle. Phases 1 (early repolarization) and 2 (plateau) have no clear counterpart in skeletal muscle, while phase 4 (diastole) corresponds to the resting potential. Modified from (Katz 2001; Rubart & Zipes 2001).

The onset of acute ischemia produces immediate electrical, mechanical, and biomedical dysfunction of cardiac muscle. In addition to the direct effect of ischemia, reperfusion after transient ischemia may cause lethal arrhythmias. At the level of the myocyte, the immediate consequences of ischemia, which include loss of integrity of cell membranes with efflux of K^+, influx of Ca^{2+}, acidosis, reduction of transmembrane resting potentials (phase 4, Fig. 5) and enhanced automacity in some tissues, are followed by a separate series of changes during reperfusion. Those of particular interest are the possible continued influx of Ca^{2+} which may produce electrical instability and neurophysiologically induced afterdepolarization as triggering responses for Ca^{2+} - dependent arrhythmias (Myerburg & Castellanos 2001).

1.4 NF kappa B and cytokines

In ischemic tissue, different inflammation factors, such as NF-□B are activated. They may play an important role in the onset of ischemic injury. The redox-sensitive transcriptional factor NF-□B plays a pivotal role in the coordinated transactivation of cytokine and adhesion molecule genes that might be involved in myocardial damage after ischemia and reperfusion (Morishita et al 1997; Valen et al 2001). In unstimulated cells, NF-□B is found in the cytoplasma and is bound to inhibitor-□B (I□B), which prevents from entering in the nuclei. When these cells are stimulated by activation signals, specific kinases phosphorylate I□B, causing its rapid degredation by proteasomes (Fig. 6). The release of NF-□B from I□B results in the passage of NF-□B into the nucleus, where it binds to specific sequences in the promoter regions of target genes. The activated form of

NF-□B is a heterodimer, which usually consists of two proteins, a p65 subunit and a p50 subunit (Barnes & Karin 1997).

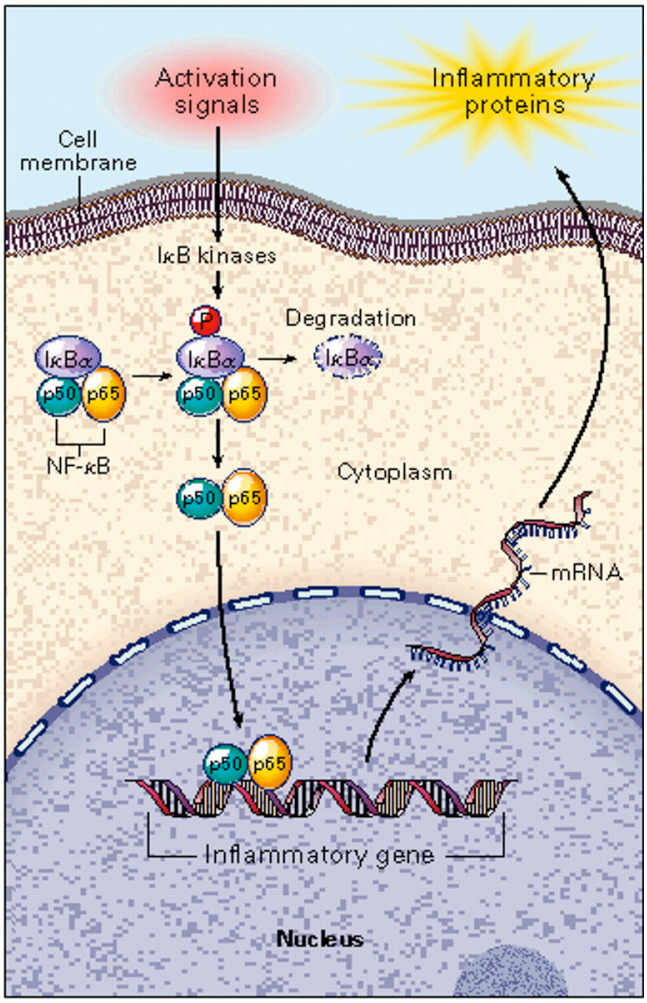


Figure 6: Schematic diagram of NF-□B activation. Activation of NF-□B involves the phosphorylation and subsequent proteolytic degradation of the inhibitory protein I□B by specific I□B kinases. The free NF-□B (a heterodimer of p50 and p65) then passes into the nucleus, where it binds to □B sites in the promoter regions of genes for inflammatory proteins such as cytokines, enzymes, and adhesion molecules. P denotes protein, and mRNA messenger RNA (Barnes & Karin 1997).

Products of the genes that are regulated by NF-□B also cause the activation NF-□B. The proinflammatory cytokines interleukin-1□ and tumor necrosis factor □ (TNF-□) both activate and are activated by NF-□B. This type of positive regulatory loop amplifies and perpetuate local inflammatory responses (Fig. 7) (Barnes & Karin 1997).

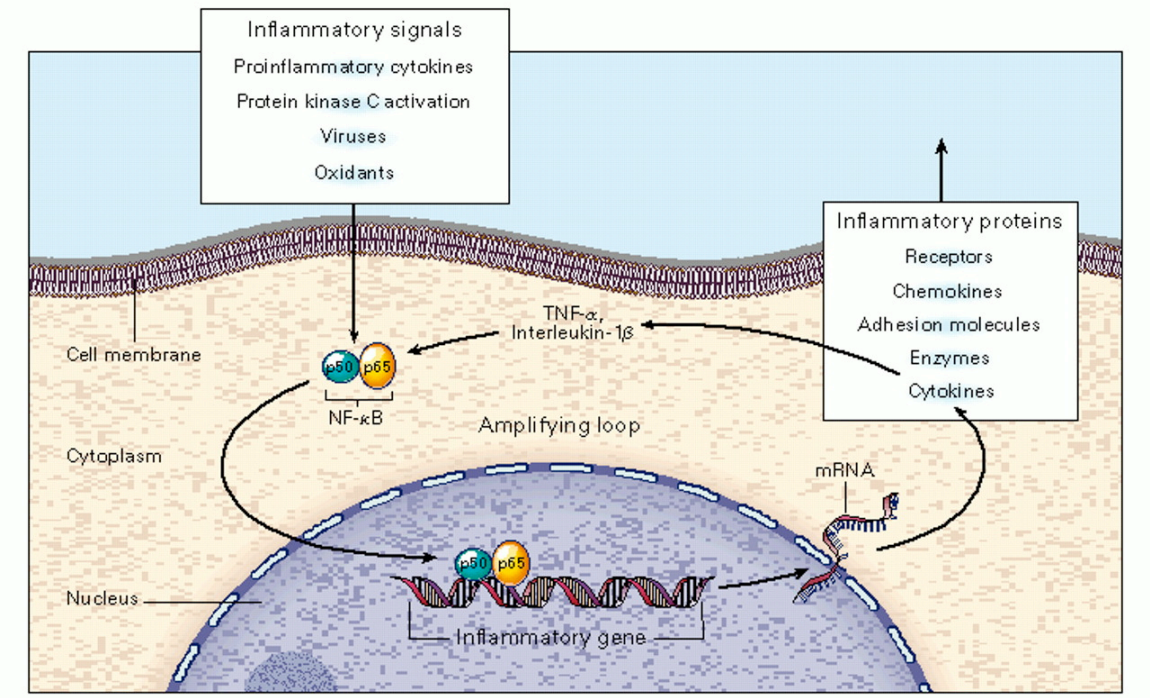


Figure 7: Schematic diagram of NF-κB as an inflammatory regulator. NF-κB may be activated by a variety of inflammatory signals, resulting in the coordinated expression of the genes for several cytokines, chemokines, enzymes, and adhesion molecules. The cytokines interleukin-1β and tumor necrosis factor α (TNF-α) both activate and are amplified by NF-κB. The abbreviation mRNA denotes messenger RNA (Barnes & Karin 1997).

Myocardial infarction provokes a biphasic activation of NF-κB, peaking after 15 min and after 3 h of reperfusion, possibly corresponding to a primary activation by reactive oxygen intermediates and a secondary activation by proinflammatory cytokines produced by the first activation. A detrimental role of NF-κB during reperfusion is suggested indirectly by functional studies of the genes it regulates: inhibition of leukocyte adhesion, of cytokines, and of chemokines protect the heart against reperfusion injury (Chandrasekar & Freeman 1997).

1.5 Losartan against reperfusion arrhythmias

Myocardial ischemia provokes arrhythmias. Additionally, sudden death is a major cause of mortality in patients with ventricular hypertrophy and heart failure (Stevenson et al 1993; Messerli 1999; Vakili et al 2001). In these patients, ventricular tachyarrhythmias, particularly VF, contribute largely to sudden death (Stevenson et al 1995). These

arrhythmias are largely due to electrophysiological abnormalities of the hypertrophied heart including prolonged action potential duration, decreased resting membrane potential, slowed conduction velocity, and heterogeneous recovery following depolarization (Aronson & Ming 1993; Stevenson et al 1995).

In this regard, it is important that the AT_1 blocker losartan has been proposed to be associated with a lower mortality than that found with the ACE inhibitor captopril in elderly heart failure patients (Pitt et al 1997). This difference appeared to arise largely from a decrease of sudden death in losartan-treated patients causing various investigators to suggest acute antiarrhythmic effects of losartan (Thomas et al 1996; Lee et al 1997). However, the effect on sudden death was not confirmed by a recent trial that was adequately sized for mortality (Pitt et al 2000). Still, various experimental studies could demonstrate acute antiarrhythmic effects of angiotensin II receptor blockers and/or of ACE inhibitors. Accordingly, in human atrial tissue, losartan significantly reduced angiotensin I-induced norepinephrine release (Rump et al 1998). In an experimental study in guinea pigs, losartan exerted antiarrhythmic effects independent of AT_1 receptor blockade (Thomas et al 1996). In another study in spontaneously hypertensive rats, losartan exerted antiarrhythmic effects in the setting of myocardial infarction (Lee et al 1997). In contrast, in rat hearts during ischemia/reperfusion, captopril was superior to losartan in reducing the incidence of irreversible VF (Ozer et al 2002). Based on the foregoing, it is controversial whether AT_1 blockers and/or ACE inhibitors exert antiarrhythmic effects, particularly in hypertrophied hearts. These hearts are particularly vulnerable to ischemia-reperfusion-induced arrhythmias because of their coronary reserve.

1.6 Dimethyl fumarate against myocardial infarction

NF-κB is a redox-sensitive transcription factor regulating a battery of inflammatory genes (Valen et al 2001). A diverse range of stimuli can activate NF-κB, suggesting that several different signaling pathways are capable of triggering the activation of this transcription factor (Marczin et al 2003). Additionally, NF-κB has been suggested to play a role in

myocardial ischemia/reperfusion injury. The process of ischemia- reperfusion injury has been postulated to be dependent on the coordinated activation of a series of cytokine and adhesion molecule genes, whose regulation involves NF-κB (Morishita et al 1997). Specifically, NF-κB levels were increased in postischemic rat myocardium and NF-κB binding activity was time-dependently increased in cultured cardiac cells in hypoxia (Chandrasekar & Freeman 1997; Kacimi et al 1998). Furthermore, transfection of NF-κB decoy oligodeoxynucleotides to myocardium significantly reduced the area of infarction (Morishita et al 1997). Finally, adenosine was recently found to prevent activation of NF-κB during ischemia (Li et al 2000), potentially explaining cardioprotective properties of adenosine related to down-regulation of TNFα.

NF-κB also plays a role in myocardial protection conferred by ischemic preconditioning. The molecules i-NOS and cyclooxygenase-2 play an important role in the cascade of ischemic preconditioning (Bolli et al 2002). The promotors of the i-NOS and the cyclooxygenase-2 genes contain cognate sequences for NF-κB. Binding of NF-κB to these promotors results in a transcriptional activation of the i-NOS and the cyclooxygenase-2 genes, which leads to cardioprotective i-NOS-derived NO and the synthesis of cardioprotective prostanoids (Bolli et al 2002). Nevertheless in the setting of myocardial infarction blocking of NF-κB shows positive effects in an experimental study (Morishita et al 1997), although the beneficial effects of ischemic preconditioning seem to be abolished.

DMF is an active ingredient of the oral antipsoriaticum Fumaderm® (Fumapharm, Switzerland) and is known to inhibit TNFα-induced activation of endothelial cells in vivo and in vitro. In human endothelial cells, DMF inhibits TNFα-induced tissue factor mRNA transcription and protein expression. It seems that DMF inhibits NF-κB induced gene transcription at the level of nuclear entry of p65 after its release from IκB (Loewe et al 2002). Thus, DMF might also inhibit NF-κB in cardiomyocytes and thus reduce myocardial infarction following ischemia and reperfusion.

2 Goal of the studies

2.1 Losartan against reperfusion arrhythmias

The goal of the first study was to assess acute antiarrhythmic effects of losartan and enalaprilat in hypertrophied rat hearts during ischemia/reperfusion. For dose-finding purposes, we determined the effects of these drugs on action potential duration in dose-response curves as well as the effects of these drugs on VF threshold in non-hypertrophied isolated perfused hearts. Subsequently, we determined the effect of these drugs on the incidence and duration of ventricular tachycardia (VT) and of VF induced by low-flow ischemia and reperfusion in hypertrophied hearts 70 days after aortic banding. Low-flow ischemia was chosen, because from a clinical point of view, low-flow ischemia is both relevant to hypertrophied hearts (due to reduced coronary reserve) and responsible for life-threatening ventricular arrhythmias (Furukawa et al 1991).

2.2 Dimethyl fumarate against myocardial infarction

The aim of the second study was to determine whether DMF might also inhibit NF-□B in cardiomyocytes and thus reduce myocardial infarction following ischemia and reperfusion.

3 Materials and methods

3.1 Losartan against reperfusion arrhythmias

3.1.1 Animals

All experiments conformed to the rules of the Swiss Federal Act on Animal Protection (1998), and were approved by the veterinary department of Basel (Switzerland). Furthermore, we performed all experiments in male Sprague Dawley rats from Iffa credo (L'Arbresle, France).

3.1.2 Aortic banding

We induced left ventricular hypertrophy by pressure overload consequent to abdominal aortic banding (coarctation) in rats. To this end, we anesthetized rats weighing 182 ± 14 (SD) g using inhalation anesthesia consisting of isoflurane (1.3%), N_2O (90 ml min^{-1}) and O_2 (30 ml min^{-1}). We isolated the abdominal aorta via lateral laparotomy and applied a tantalum clip (Horizon™ ligating clip, Weck closure systems™, USA) with constant inner diameter to the aorta proximal to the branching of the A. renalis (Fig. 8 and Fig. 9).

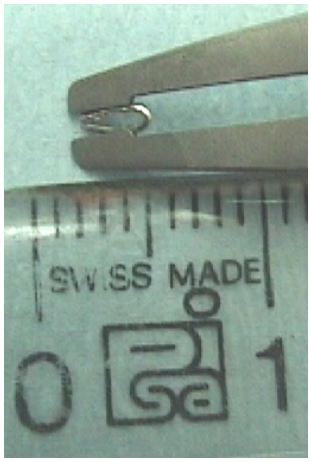


Figure 8: Clip as applied to the aorta

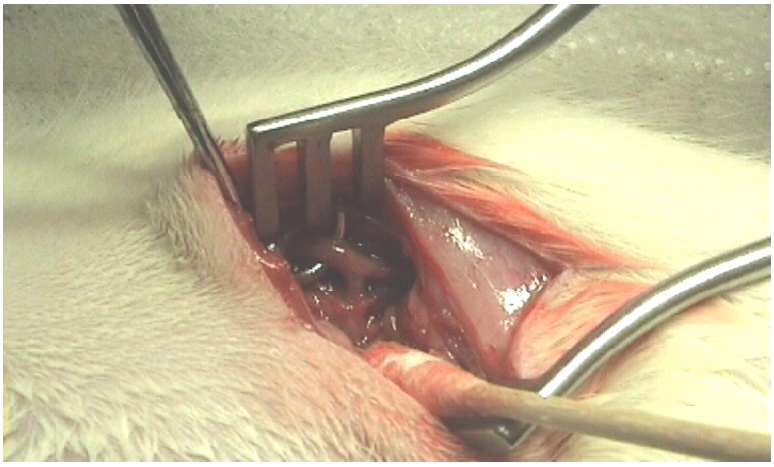


Figure 9: Preparation of the ascending aorta of a rat. We applied the clip proximal to the branching of A. renalis.

Sham-operated animals were treated the same way, however no clip was applied. We kept the rats at a normal diet and water ad libitum for 70 days to develop left ventricular

hypertrophy. Subsequently, we confirmed left ventricular (LV) hypertrophy by repeated *in vivo* magnetic resonance imaging (in 3 rats each) (Ziegler et al 2002). Those experiments were performed in the Biocenter of the University of Basel. Additionally, we confirmed LV hypertrophy *ex vivo* by determining the ratio wet heart weight/body weight (of all rats). Seventy days after surgery, we isolated the hearts and perfused them to assess acute antiarrhythmic effects of losartan and enalaprilat during ischemia/reperfusion.

3.1.3 Isolated perfused rat heart

3.1.3.1 Introduction

Oscar Langendorff first described the isolated perfused mammalian heart in 1895 (Langendorff 1895). Langendorff's contributions to cardiovascular physiology reach far beyond the demonstration that the mammalian heart can be kept alive outside the body. One of Langendorff's hypotheses was that the heart receives its nutrients through the coronary circulation, that oxygen is extracted from the blood as it passes through the coronaries, and those modifications in the coronary circulation result in changes in contractile function of the heart. Langendorff proved his hypothesis by tying the aorta of a cat heart to a cannula filled with defibrinated blood at a hydrostatic pressure sufficient to keep the aortic valve closed just as in vivo during diastole when the blood is spread in the coronary arteries. After passing the aorta, the blood takes its way through the orifices of the coronary arteries into the coronary circulation and supplies the heart with the necessary nutrients and oxygen. After flowing through the coronary vascular system, the blood passes the coronary sinus and flows into the right atrium. The blood can easily leave the preparation via the openings of the caval veins or the pulmonary artery. Under these conditions the heart continued beating for more than three hours (Taegtmeyer 1995). Already in 1898 one of Langendorff student's demonstrated that blood could be substituted by a glucose-containing saline medium as nutrient solution for the heart (Rusch 1898). Today, more than 100 years later, Langendorff's method is still used by many investigators, including us, examining the function and metabolism of the heart.

As an advantage of this model over *in vivo* experiments, the external variables like temperature, pH, ion concentration, energy substrates, and drugs can easily be standardized. Working on the isolated heart means less severe animal experiments and is therefore more ethical.

3.1.3.2 The Langendorff perfusion system according to Schuler

We used a Langendorff perfusion system according to Schuler (Hugo Sachs Elektronik-Harvard Apparatus, Mach-Hugstetten, Germany) for our experiments. Figure 10 shows the experimental set-up of the used system. This system allows perfusion with constant pressure or with constant flow.

The pump transports the perfusate into the temperated oxygenator (37°C) where the perfusate is heated and supplied with oxygen. The sling disc disperses the perfusate as a thin film over the entire surface of the oxygenator and allows good oxygenation and heating. This system enables oxygenation of foaming solutions as perfusate containing albumin without interfering foaming. The perfusate gains a partial oxygen pressure of about 665 mm Hg and a pH of 7.4 (Zink et al 1998). Oxygenation is provided by 95% O_2 and 5% CO_2. The surplus gas goes into a water filled column (Gottlieb valve). The immersing depth of the Gottlieb valve is responsible for the perfusion pressure and therefore provides the constant perfusion pressure of the Schuler system. Warm-blooded animals' perfusion pressure *in vivo* corresponds to a large extent to their diastolic aortic pressure, which normally varies between 70 and 90 mm Hg. Thus the recommended perfusion pressure for the isolated rat heart is around 70 mm Hg (Zink et al 1998). To maintain a constant perfusate level, a contact electrode measuring and adjusting the perfusate level, controls a connected circulating pump. The heart is connected by a perfusion canula to the oxygenator and maintained at 37°C by immersion into an adjustable heating chamber containing warmed perfusion solution.

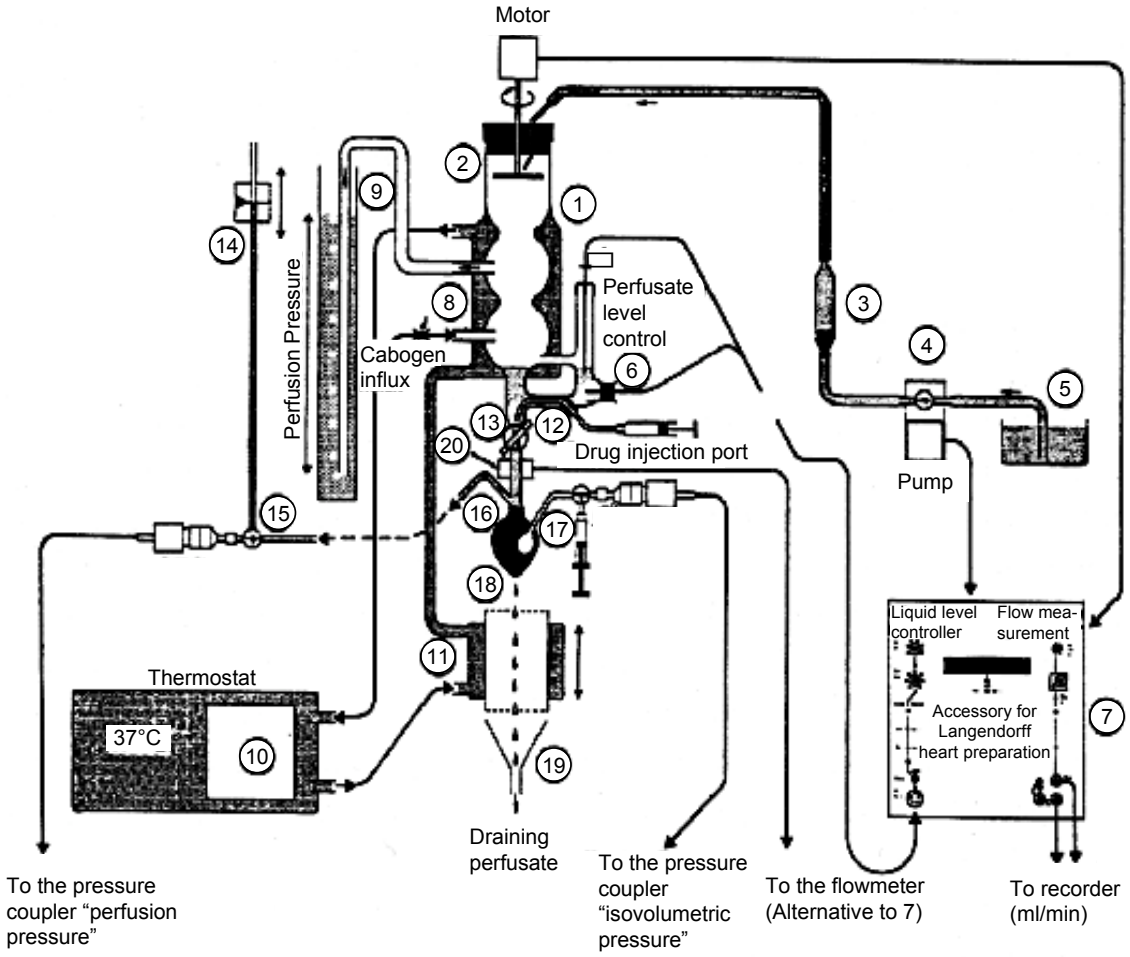


Figure 10: A typical Langendorff set-up according to Schuler (Döhring & H. 1988).

1. Oxygenator
2. Sling disc
3. Non-return valve
4. Gear pump for nutritive solution
5. Supply vessel for nutritive solution
6. Contact electrode for perfusate level control
7. Electronic control for measurement of flow
8. Inlet and outlet for gas, e.g. carbogen
9. Adjustment valve (Gottlieb valve) for perfusion pressure (pressure relief valve)
10. Thermostat
11. Heart recipient
12. Tube catheter for drug addition with injection syringe
13. Stopcock
14. Water manometer or mechanoelecrtic pressure transducer for perfusion pressure
15. Three-way cock
16. Aortic canula with side nozzle
17. Ballon catheter with pressure transducer for isovolumetric measurement of ventricular pressure
18. Heart
19. Collecting funnel for nutritive solution dripping of the heart
20. Flow probe

3.1.3.3 The perfusion solution

The isolated perfused heart still needs the necessary nutrients and oxygen as it needs *in vivo*. Oscar Langendorff perfused the heart with blood or blood diluted with Ringer solution. As this method requires blood from donor animals, most of the time blood is substituted by a perfusion solution defined by Krebs and Henseleit (Sutherland & Hearse 2000). This perfusion solution was described in 1932 by Sir Hans Adolf Krebs and Kurt Henseleit (Krebs & Henseleit 1932) and was supposed to mimic the key ionic content of blood. Unfortunately Krebs and Henseleit failed to take into account the fact that much of the calcium in blood is bound to proteins and the realistic plasma ionized calcium concentration is approximately half of the recommended value of 2.5 mM. Therefore we used a filtered (pore size 0.65 µm, Millipore, Volketswil, Switzerland) nonrecirculating modified Krebs-Henseleit buffer containing (in mM) NaCl 117, KCl 4.3, $MgCl_2$ 1.2, $CaCl_2$ 2.0, $NaHCO_3$ 25, EDTA 0.5 and glucose 15 at pH 7.4 at a constant perfusion pressure of 80 mm Hg for the experiments described in my thesis. To support the large energy requirements of cardiac contractile function, we used glucose as substrate. Certain investigators add albumin to the perfusion solution to prevent edema due to low colloidal osmotic pressure. The buffer used for my experiments did not contain albumin to avoid losartan, a drug used in the study, binding to albumin (Christ 1995). The different drugs used in the study we added to the perfusate. We saturated the buffer with 95% O_2/5% CO_2 ensuring a minimal pO_2 of 550 mm Hg.

3.1.3.4 Perparation of the heart

We anaesthetized the rat with an intraperitoneal injection of 30 mg kg^{-1} sodium pentobarbital (Nembutal, Abbott Laboratories, Chicago, IL, USA). After midline sternotomy, we clamped the ascending aorta at the aortic arch and cut out the heart still attached to the lungs within a few seconds. Immediately we immersed the heart into ice-cold Krebs-Henseleit perfusate to reach cardioplegia. After a short preparation of the aorta, we cannulated the aorta to the perfusion canula and tied it together with a surgery string (Pearsall Sutures Ltd., Taunton, United Kingdom). The perfusion with Krebs-

Henseleit buffer at 37°C and a perfusion pressure of 80 mm Hg began without delay and therefore the heart started beating again. In general, the whole procedure lasted less than 60 sec. After making a small incision into the right ventricular outflow tract, we ligated the pulmonary vessels and completely removed the hilus and the lung tissue. A superficial incision on the left ventricle allowed the insertion of a pressure catheter into the left ventricle to measure left ventricular pressure. We placed the catheter through the mitral valve into the left ventricle and tied it with a surgery string at the appendix (Zaugg et al 1996b). During all experiments, we immersed the hearts in perfusate maintained at exactly 37.0°C (Zaugg et al 1996b) preventing bradycardia due to cardiac cooling (Curtis 1998). Figure 11 shows a prepared isolated rat heart whereas Figure 12 shows the cross section of a prepared heart.

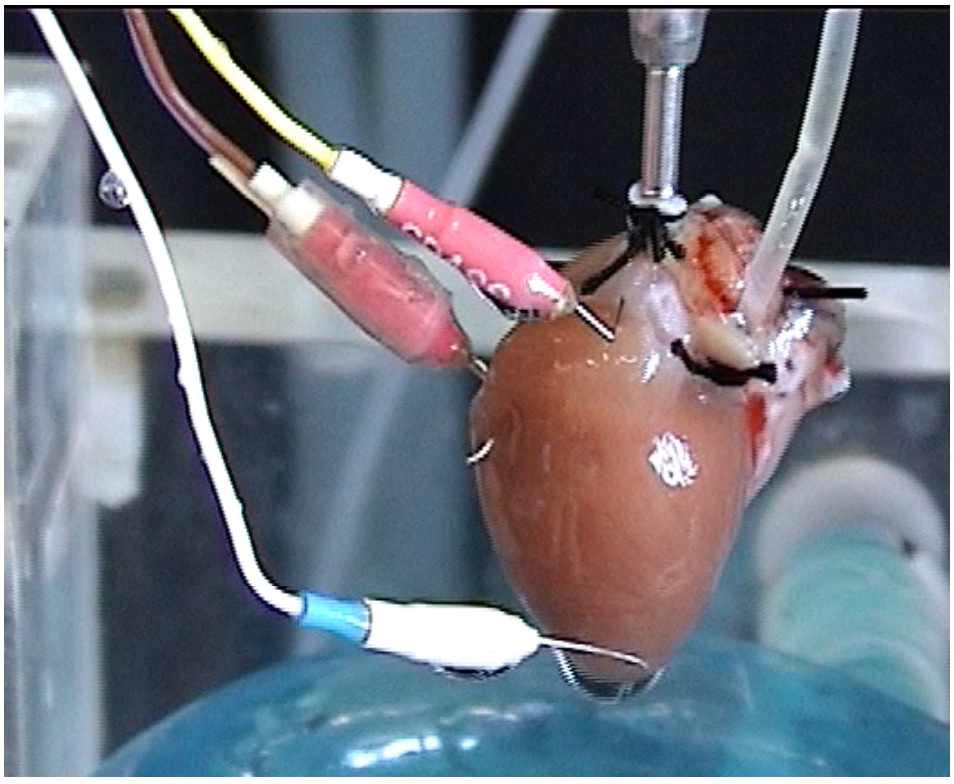


Figure 11: Isolated rat heart with pressure catheter, ECG electrodes (white and yellow wires), and pacing electrodes (purple and brown wires).

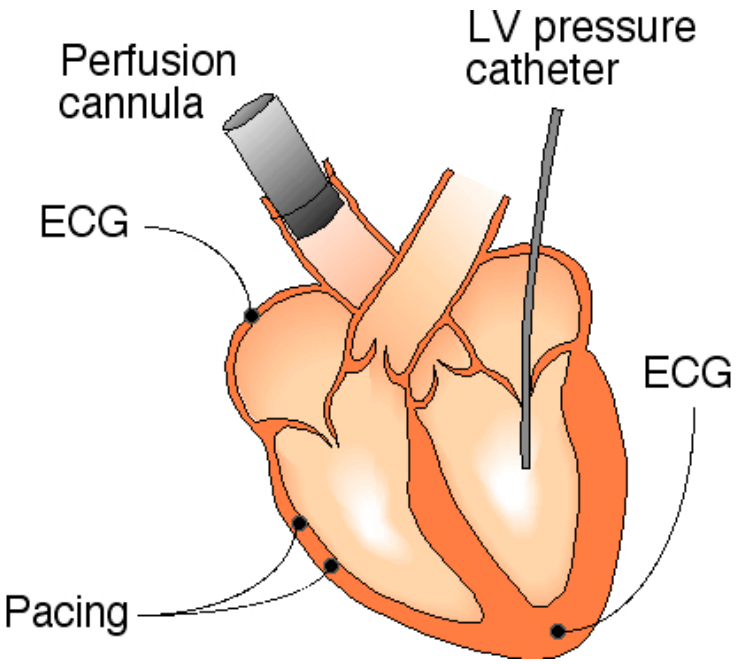


Figure 12: Cross-section of a prepared rat heart showing the aorta fixed to the perfusion cannula as well as the position of the pressure catheter, the ECG and the pacing electrodes (Artwork by C. E. Zaugg, modified).

3.1.4 Experimental protocols

We performed dose-finding experiments of losartan and enalaprilat (dose-response curves for action potential duration and VF threshold experiments) in isolated non-hypertrophied hearts from non-operated rats weighing 353 ± 52 g. Acute antiarrhythmic effects of these drugs during low-flow ischemia and reperfusion, however, we assessed in isolated hypertrophied hearts 70 days after aortic banding surgery. Furthermore, in all experiments, a 15-min stabilization period preceded any drug administration or pacing protocol.

3.1.4.1 Monophasic action potentials

To measure monophasic action potentials, two electrodes are needed. One electrode is pressed against the epicardium while the other electrode merely touches the nearby epicardium. This allows the detection of phasic electrical changes of the cardiac cycle.

New contact electrode catheters simplify the measurement as both electrodes are attached to the catheter (Franz 1999). We recorded monophasic action potentials using two contact electrode catheters (Ag-AgCl electrode, model 225, Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) on the epicardium of the left ventricle (Fig. 13) and of the right ventricle. For reliable results it was important that the position of the electrodes and the contact pressure stayed the same throughout the experiments. Pilot experiments using the decoupler 2,3-butanedione monoxime to avoid motion artifacts did not show better results than beating hearts. Although 2,3-butanedione monoxime could inhibit contraction, the quality of the digitized readout did not improve. We optained best results by placing the electrodes at a part of the heart with little contraction movement. We recorded a digitized readout at 1 kHz sampling rate using a PowerLab 4/20 data acquisition system running Chart software. We confirmed the validity of monophasic action potential recording by determining the effect of increasing heart rate on monophasic action potential duration at 90% repolarisation ($MAPD_{90\%}$, Fig. 13). Accordingly, $MAPD_{90\%}$ consistently decreased when heart rate was increased from 100 to 300 beats per min (at 30°C to allow for heart rates <200 min^{-1}, Fig. 14). For experiments determining the effects of losartan or enalaprilat on $MAPD_{90\%}$ we held heart rate constant at 240 beats per min (at 37°C).

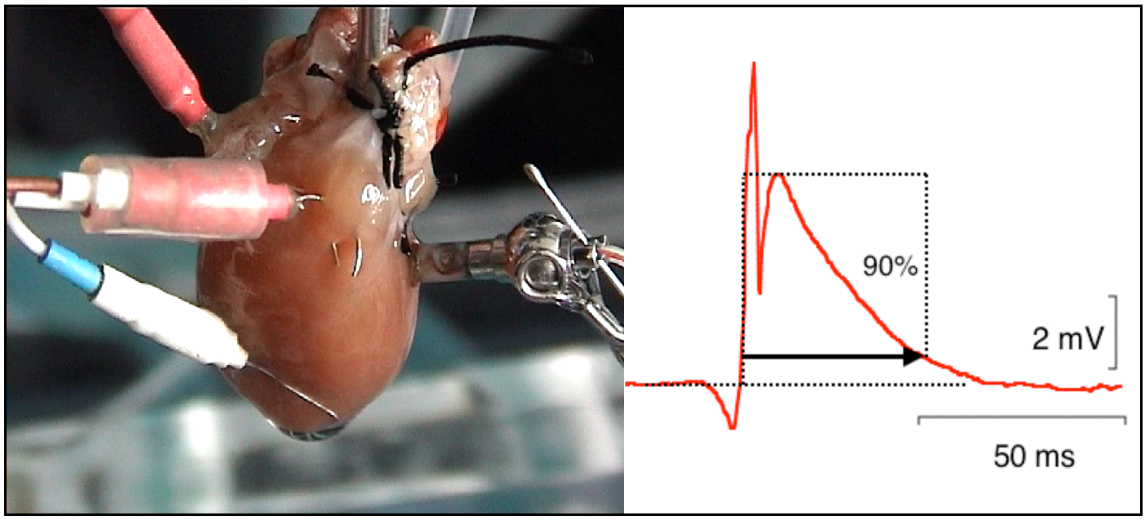


Figure 13: Isolated rat heart with monophasic action potential measuring electrode (left) and determination of $MAPD_{90\%}$ (right).

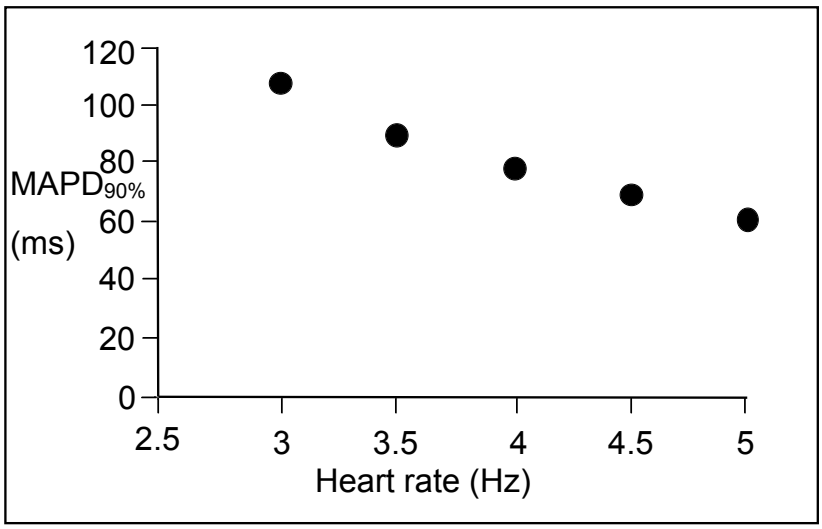


Figure 14: Effect of heart rate on $MAPD_{90\%}$ in normal hearts (recorded at 30°C).

3.1.4.2 Dose-response curves

To find suitable concentrations for subsequent experiments and to assess acute electrophysiologic effects of the drugs, we performed dose-response curves of losartan (1 nM to 1 mM, n=4) and of enalaprilat (1 nM to 1 mM, n=4) studying $MAPD_{90\%}$. As positive control we used the potassium channel blocker amiodarone (10 □M), which has been shown to prolong action potential duration (Rochetaing et al 2001). The dose-response curves were to select a concentration of losartan and enalaprilat for subsequent experiments (VF threshold and ischemia/reperfusion experiments).

3.1.4.3 VF threshold

We performed VF threshold determination using a train-of-pulses method at increasing voltage to scan the vulnerable period of repolarisation (Zaugg et al 1996a). Specifically, the stimulator mode of a PowerLab 4/20 data acquisition system (1 ms monophasic square wave pulses at 4 V) provoked a constant heart rate at 200 ms pulse interval. After 30 regular pulses, it generated a train-of-pulses (100 Hz, 250 ms duration) at increasing voltage. We increased voltage starting at 0.25 V in 0.25-V increments (until 1 V) and in 0.5-V increments (until 10 V) until VF occurred. After each train-of-pulses, we stopped pacing for 2.5 s to allow detection of VF. We defined the VF threshold as the mean voltage (in V) of at least three successive measurements, which were reproducible within

limits of ±15%. ECG waves of irregular morphology without corresponding effective left ventricular pressure for longer than 1 s were detected as VF (Zaugg et al 1996a). We determined VF thresholds before and after perfusion with losartan (1 μM, n=6; for choice of concentration see *Results*), enalaprilat (10 μM, n=5), or vehicle (control, n=6). As a positive control, we determined VF thresholds before and after perfusion with the sodium channel blocker lidocaine (3 μM). To reduce VF threshold variability, the pacemaker electrodes were coated and held in position by polyethylene tubes to ensure a constant implantation depth of 2 mm (uncoated electrodes) and a consistent distance of 5 mm from each other (Zaugg et al 1996a). Thereby, we kept the spatial separation and anatomical position of electrodes on the heart consistent, as recommended for reproducible VF threshold determination (Van Tyn & MacLean 1961).

3.1.4.4 Arrhythmias induced by low-flow ischemia and reperfusion

We randomly assigned the hypertrophied hearts to one of three groups: Control (receiving only Krebs-Henseleit buffer (vehicle), n=10), losartan (1 μM, n=9) or enalaprilat (10 μM, n=9). Hearts of sham-operated rats (n=18) were perfused with the vehicle only. After a drug-free stabilization period of 20 min, we administered losartan or enalaprilat 15 min before low-flow ischemia until the end of the experiment. Low-flow ischemia lasted for 60 min. We induced low-flow ischemia by reducing perfusion pressure from 80 to 15 mm Hg, reducing coronary flow by ≈90%. To prevent bradycardia and asystole during low-flow ischemia we paced the hearts (as recommended by (Curtis 1998)) at 300 beats per min *via* a pair of platinum pacemaker wires implanted in the right ventricular free wall and connected to a pulse generator (Grass SD 5, Grass Instruments, Quincy, MA, USA). We stopped pacing before normalizing the perfusion pressure to 80 mm Hg and subsequent reperfusion lasted for 60 min.

3.1.5 Hemodynamic variables

3.1.5.1 Coronary flow

An inline flowprobe (Transonic 2N) connected to a transit time flowmeter (Transonic TTFM-SA type 700, Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) measured coronary flow within the aortic canula. This inline flowprobe allowed continuous and reliable recording of coronary flow (in mL/min) throughout the experiments.

3.1.5.2 Left ventricular pressure

We measured LV pressure by a fluid-filled polyethylene catheter inserted through the left atrial appendage into the LV cavity. The catheter was connected to a pressure transducer (MLT1050 Pressure transducer, AD Instruments, Castle Hill, Australia). For reliable recordings, it was important that the membrane of the pressure transducer was at the same height as the heart. A PowerLab 4/20 data acquisition system (AD Instruments, Castle Hill, Australia) connected to a Macintosh computer running Chart software (AD Instruments, Castle Hill, Australia) recorded a digitized readout of the LV pressure at 400 Hz sampling rate throughout the experiment. We defined LV developed pressure as the difference between systolic and diastolic values of LV pressure.

3.1.5.3 ECG

To record a bipolar epicardial electrocardiogram (ECG), we placed a pair of electrodes (0.28 mm diameter, 2-3 mm contact length) on the right appendage and apex. The electrodes were connected to a PowerLab 4/20 data acquisition system controlled by a Macintosh computer running Chart software, which allowed to record a digitized readout of the ECG at 400 Hz sampling rate throughout the experiment.

3.1.5.4 Heart rate

The digitized readout of the ECG allowed the calculation of the heart rate (in beats/min [bpm]) using Chart software.

3.1.6 Analysis of ventricular arrhythmias

We analyzed ventricular arrhythmias according to the Lambeth Conventions (Walker et al 1988). Therefore, we categorized arrhythmias as ventricular tachycardia (VT, run of four or more consecutive ventricular premature beats with corresponding effective LV pressure) or ventricular fibrillation (VF, ECG waves of irregular morphology without corresponding effective LV pressure). We did not analyze sustained and spontaneously reverted VF separately, and terminated VF persisting longer than 30 sec by a bolus of 0.25 mg lidocaine hydrochloride injected into the perfusion canula proximal to the aorta. After termination of VF, we washed out lidocaine within 5 min (Zaugg et al 1996a) and re-included the corresponding heart in the arrhythmia analysis until the end of the experimental protocol. This way, we avoided potential study bias due to early exclusion of experiments after sustained VF.

3.1.7 Exclusion criteria

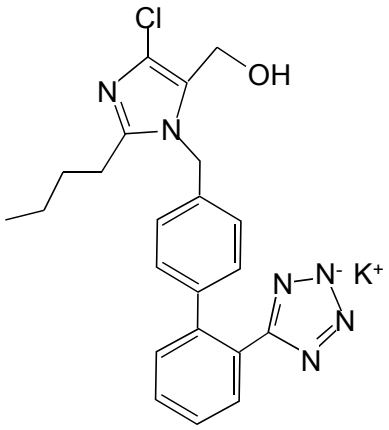
We decided upon the following criteria for the exclusion of an experiment:

- Incorrect position of the aortic clip (checked post mortem)
- VT or VF during stabilization period
- Unstable left ventricular pressure signal
- Perforation of the aorta
- Technical problem during recording of Chart file (computer crashes)

3.1.8 Characterization of drugs

3.1.8.1 Losartan

Chemical structure:



Chemical formula: $C_{22}H_{22}ClKN_6O$

Molecular weight: 461 g/mol

Manufacturer: Merck Research Laboratories, Rahway, New Jersey, USA

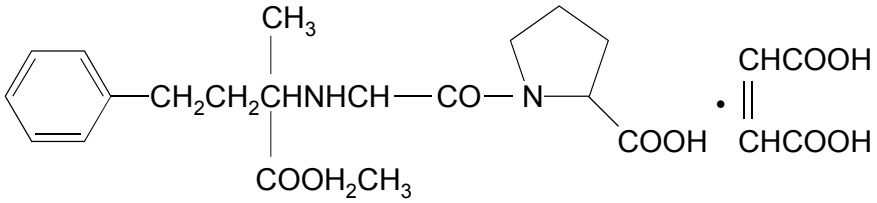
Solubility: Soluble in water or saline

Stability: Stable at room temperature

Function: Angiotensin II receptor type 1 (AT_1) blocker

3.1.8.2 Enalaprilat (Enalapril maleat)

Chemical structure:



Chemical formula: $C_{20}H_{28}N_2O_5 \cdot C_4H_4O_4$

Molecular weight: 492.53 g/mol

Manufacturer: Merck Research Laboratories, Rahway, New Jersey, USA

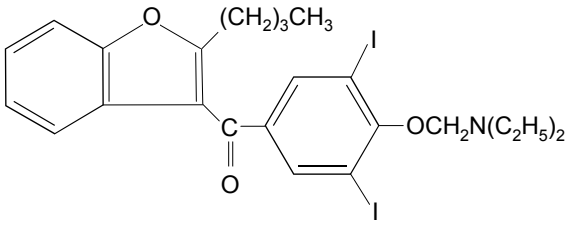
Solubility: Soluble in water or saline

Stability: Stable at room temperature

Function: ACE inhibitor

3.1.8.3 Amiodarone

Chemical structure:



Chemical formula: $C_{25}H_{29}I_2NO_3$

Molecular weight: 681.8 g/mol

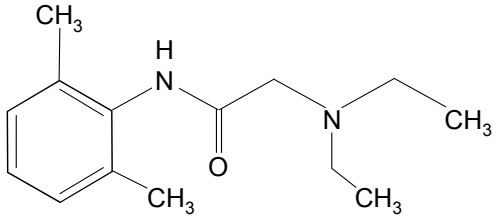
Manufacturer: Sanofi-Synthelabo, Switzerland (Cordarone® Injection solution 50 mg/ml)

Function: Potassium channel blocker

We added Cordarone® Injection solution 50 mg/ml to the Krebs-Henseleit buffer.

3.1.8.4 Lidocain

Chemical structure:



Chemical formula: $C_{14}H_{22}N_2O$

Molecular weight: 234 g/mol

Manufacturer: Sintetica, Switzerland (Rapidocaine® 1%)

Function: Sodium channel blocker

We diluted Rapidocain ® 1% 1:10 and injected the solution into the perfusion canula proximal to the aorta.

3.1.9 Evaluation and statistical analysis

Normal distribution of numerical variables (magnetic resonance data, ratio wet heart weight/body weight, coronary flow, LV developed pressure, $MAPD_{90\%}$, and VF threshold) was confirmed by Shapiro-Wilk test. Consequently, these variables were expressed as mean ± SD and compared among groups by one way analysis of variance (ANOVA). Dose-response curves of losartan and enalaprilat on $MAPD_{90\%}$ were analyzed by repeated-measures ANOVA. The incidence of VT and of VF were evaluated on the digitized ECG and pressure readouts and compared among groups by chi-squared analysis. To obtain a more sensitive measure of potential antiarrhythmic effects, an overall duration of ventricular tachyarrhythmias was calculated and compared among groups. To this end, the duration of VT and of VF was pooled during low-flow ischemia and during reperfusion for each experiment. Because of non-Gaussian distribution, the duration of ventricular tachyarrhythmias was expressed as median with interquartile range (distance between 25^{th} and 75^{th} percentile) and compared among groups by Kruskal-Wallis test followed by Dunn's test. One hypertrophied heart demonstrating non-sustained VT before low-flow ischemia was excluded from analysis. Testing for far outliers was performed according to the method of Velleman and Hoaglin (Velleman & Hoaglin 1981), excluding one experiment in the sham-operated and one in the losartan-treated hypertrophied hearts in the analysis of arrhythmias during ischemia and reperfusion. Statistical computations were done using Prism software (GraphPad, San Diego, CA, USA; version 3.0a). In an approximation of sample size determination for this study, 9 rats in each group had 90% power to detect a biologically meaningful difference of at least 20% in most variables assuming a SD of 15% and a 0.05 two-sided significance level. The study was, however, not meant and powered to demonstrate differences in the incidence of VF or of VT. For all statistical analyses, the null hypothesis was rejected at the 95% level, considering a two-tailed $p<0.05$ significant.

3.2 Dimethyl fumarate against myocardial infarction

3.2.1 Animals

All experiments conformed to the rules of the Swiss Federal Act on Animal Protection (1998), and were approved by the veterinary department of Basel (Switzerland). Furthermore, we performed all experiments in male Sprague Dawley rats from Iffa credo (L'Arbresle, France).

3.2.2 Animal model of acute myocardial ischemia and reperfusion

We used a rat model of reversible myocardial ischemia and reperfusion (Fig. 15) (Barbosa et al 1996). Accordingly, we anesthetized male Sprague-Dawley rats weighing 200-250 g in a chamber using 3% isoflurane (Forene®, Abbott AG, Baar, Switzerland) in air. Subsequently, we intubated the rats and ventilated them on a Harvard rodent respirator (Harvard rodent ventilator, model 683, Harvard apparatus, Holliston, USA). A continuous general anesthesia with 1.3% isoflurane in O_2 (30 ml/min) / N_2O (90 ml/min) at a tidal volume of 2 ml and a respiratory rate of 60 per min was thus enabled. We performed a midline sternotomy and immediately electro-coagulated all bleeding vessels to minimize blood loss. After opening the pericardium, we placed a reversible coronary artery snare occluder (Ti-Cron 7-0, 3280-01 2xCV-301, Sherwood-Davis & Geck, St. Louis, USA) around the proximal left coronary artery. To make occlusion possible we placed polyethylene tubing (Intramedic®, Clay adams®, PE-90, ID 86 mm, Becton Dickinson, Sparks, USA) around the suture and closed the occluder gently to ensure that no damage occurred to the artery (Fig. 16). To confirm the correct position of the occluder, we performed a brief test occlusion. The presence of myocardial ischemia we confirmed by regional cyanosis, reperfusion we verified by hyperemia after releasing the snare. Finally, we placed a pair of ECG electrodes on the sternum and the abdomen. After a stabilization time of 20 min, we started the experimental protocol described below.

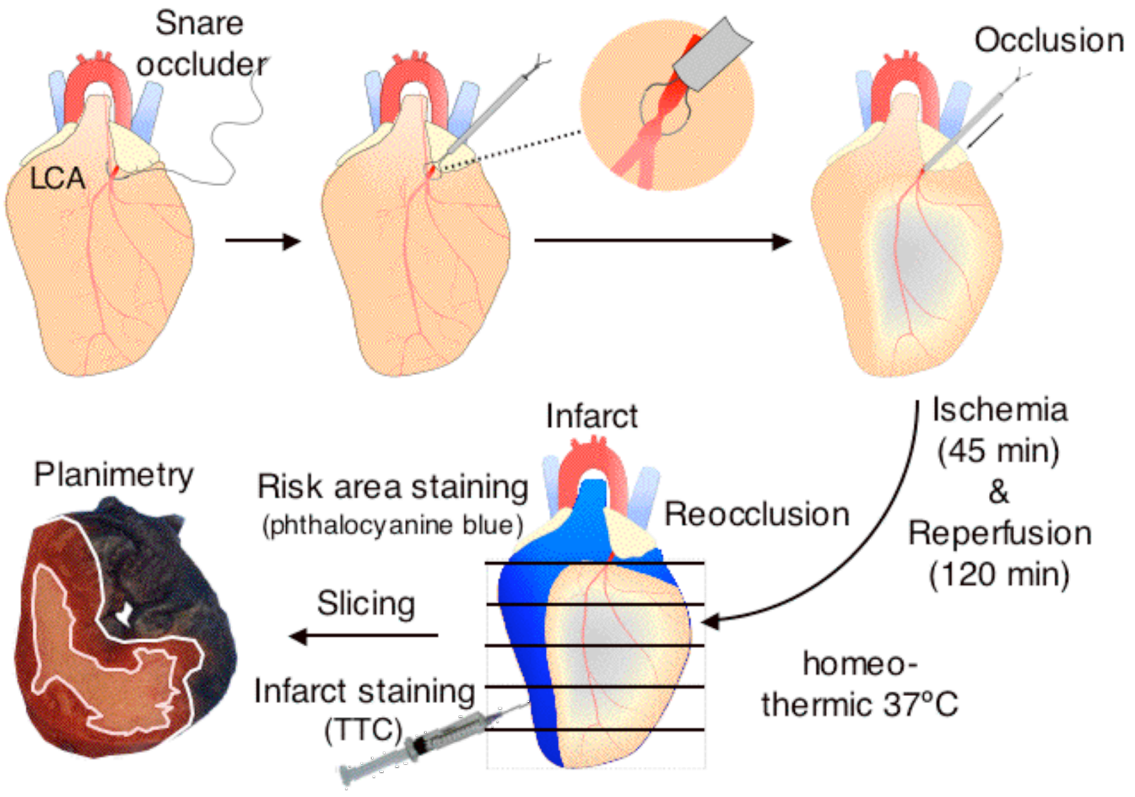


Figure 15: Scheme of the rat model of acute ischemia and reperfusion (Artwork by C. E. Zaugg).

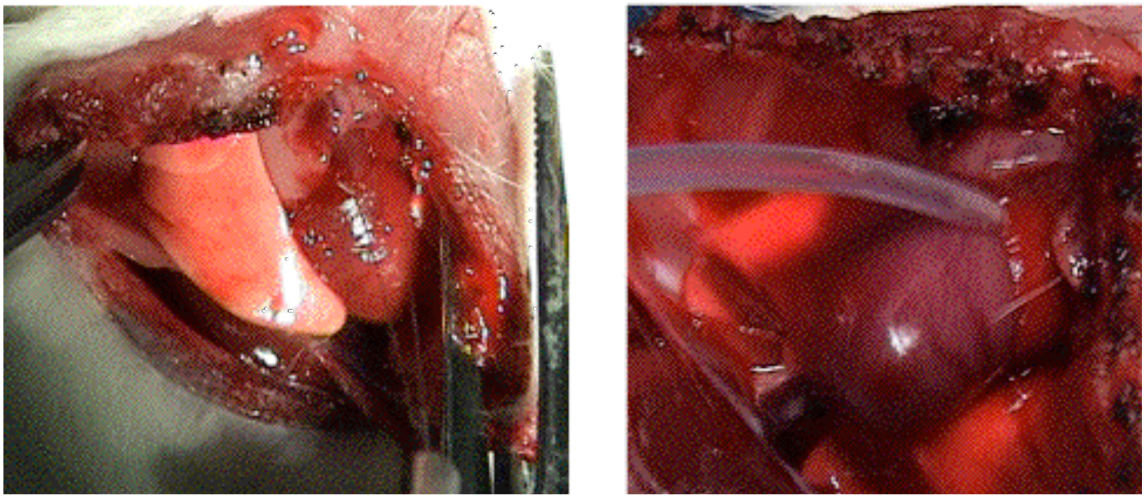


Figure 16: Different stages of left coronary artery occlusion: after placing the occluder (left), and after some minutes of ischemia (right).

3.2.3 Variables

3.2.3.1 Infarct size

Infarct size was determined using a double staining technique (Barbosa et al 1996). After 120 min of reperfusion, the left coronary artery was re-occluded and phthalocyanine blue dye (W-4123, Engelhard, Iselin, USA) was injected into the left ventricular cavity, causing dye to perfuse the non-ischemic region of the left ventricle, and leaving the ischemic region unstained. Then the heart was removed, rinsed of excess blue dye, trimmed off right ventricular and atrial tissue, cut transversely into 2-mm thick slices, and incubated in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Fluka, Switzerland) for 15 min. TTC stained viable myocardium bright red but did not stain necrotic myocardium. This method has been shown to reliably distinguish necrotic myocardium from viable myocardium (Reimer & Jennings 1992). After staining, the left ventricular sections were fixed in a 4% formalin solution. Then, the slices were weighed and scanned both sides of each slice. The ischemic risk area (unstained by phthalocyanine blue dye) and the infarcted area in each section (unstained by TTC) were outlined on the digital images, measured by planimetry, averaged from both sides of each slice, and multiplied by the weight of the tissue of that slice. For planimetry, the operator was blinded regarding the group assignment of the experiment. Infarct size was expressed both as a percentage of total left ventricular mass and as a percentage of the ischemic risk area.

3.2.3.2 Electrocardiogram, arrhythmias, heart rate, and QT interval

A standard lead II electrocardiogram according to Einthoven was recorded. The leads were connected to PowerLab 4/20 (ADInstruments, Castle Hill, Australia) to record a digital ECG continuously during the experiment at a sampling rate of 200 Hz. On the digitized ECG heart rate before, during, and after ischemia was analyzed. Finally, QT intervals before ischemia were analyzed. The end of the QT interval is determined by laying a tangent at the inflection point. The interval ends where the tangent intersects the

baseline. In order to correct for heart rate on the QT interval a correction formula developed by Bazett was used in (observed QT interval is divided by the square root of the RR interval). Although Bazett's formula is the most common correction formula, it is optimized for human heart rate. It is said to lead to some overcorrection at higher heart rates and undercorrection at lower heart rates. Therefore the linear regression analysis described in the Framingham Heart Study (QTc = QT + 0.154 x (1 – RR)) was also used (Brouwer et al 2003).

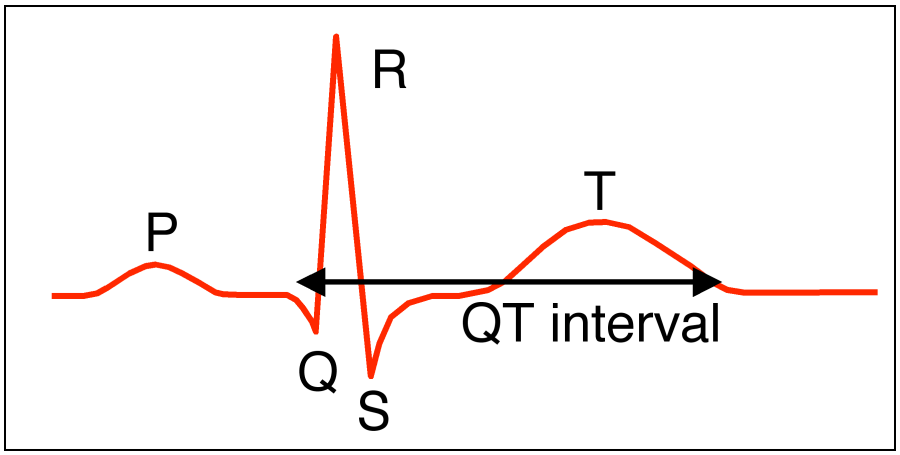


Figure 17: ECG to demonstrate the determination of the QT interval

3.2.3.3 Body temperature

Throughout the experiments, core body temperature of the rats was monitored by a rectal thermometer and maintained the body temperature of rat at 37.0±0.1°C using a Homeothermic Blanket System (Harvard Apparatus / Hugo Sachs Electronics, Germany). The thermometers were connected to a PowerLab 4/20 data acquisition system controlled by a Macintosh computer running Chart software, which allowed the recording of a digitized readout of the temperature throughout the experiment.

3.2.4 Experimental protocol

The rats were randomly assigned in a blinded design to one of three groups (Fig. 18): a) to the vehicle group receiving only the vehicle (DMSO and water; necessary to dissolve DMF), or b) to the DMF group receiving 10 mg/kg body weight DMF (dissolved in DMSO and water), or c) to the positive control group receiving the vehicle plus ischemic

preconditioning (known to reduce infarct size) (Barbosa et al 1996). DMF and the vehicle, respectively were administered i.v. into tail vein as boli (5 ml/kg)(Diehl et al 2001) 90 min before ischemia (under general anesthesia using 3% isoflurane in air) as well as immediately before ischemia. Ischemic preconditionining was induced by two times 5-min episodes ischemia (induced by left coronary artery occlusion) each followed by 5 min of reperfusion (induced by releasing the snare). To determine the effect of DMF on infarct size, all rats were subjected to a 45-minute period of ischemia by left coronary artery occlusion followed by 120 min of reperfusion, and then assessed for infarct size. To assess the effects of DMF on ischemia- and reperfusion-associated ventricular arrhythmias, a digital bipolar epicardial electrocardiogram (ECG) was recorded continuously throughout the experiment. The experiments were performed (except pilot experiments) pairwise, i.e. two experiments in parallel.

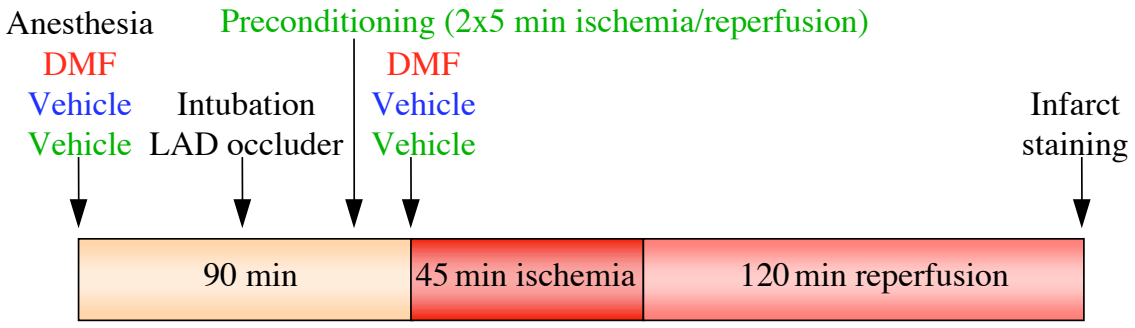


Figure 18: Experimental protocol

3.2.5 Control experiments in the isolated perfused rat heart

To exclude the possibility that beneficial properties of DMF or of its main metabolite, methyl hydrogen fumarate (MHF) were due to acute hemodynamic or electrophysiologic effects, the acute effects of DMF and MHF on heart rate, coronary flow, left ventricular developed pressure, and duration of monophasic action potentials at 90% repolarisation ($MAPD_{90\%}$) were assessed in dose-response curves in isolated perfused rat hearts in vitro.

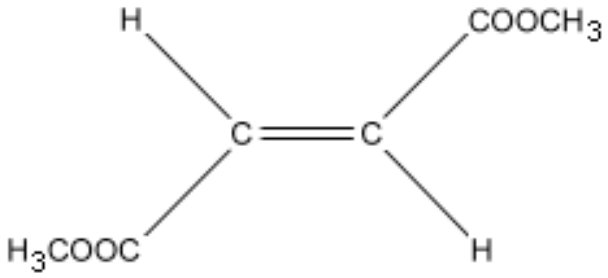
3.2.5.1 Dose-response curves of DMF and MHF

Dose-response curves of DMF (0.1 µM to 1000 µM, n=9) and of MHF (0.1 µM to 1000 µM, n=9) were performed studying heart rate, coronary flow, left ventricular developed pressure, and $MAPD_{90\%}$. DMF and MHF were perfused consecutively, the sequence of which was switched for each experiment. Each concentration of DMF or of MHF was perfused for 10 min with a wash-out period of 20 min in between. To dissolve DMF a Krebs-Henseleit buffer containing 0.004% DMSO was used. MHF was dissolved in the standard Krebs-Henseleit buffer. To rule out effects of the vehicle of DMF, 0.004% DMSO was administered alone prior to DMF perfusion.

3.2.6 Used solutions

3.2.6.1 DMF

Chemical structure:



Chemical formula: $C_6H_8O_4$

Molecular weight: 144 g/mol

Manufacturer: Fumapharm, Muri, Switzerland

Stability: solid: stable; solution: should be prepared daily

Function: Antipsoriatic agent, probably due to interaction with nuclear factor □-B

Intravenous formulation of DMF

We weighed 20.0 mg of DMF into a 10 ml volumetric flask and added 200 µl dimethyl sulfoxide (DMSO). Then we filled up the volumetric flask with water up to approx. 9 ml.

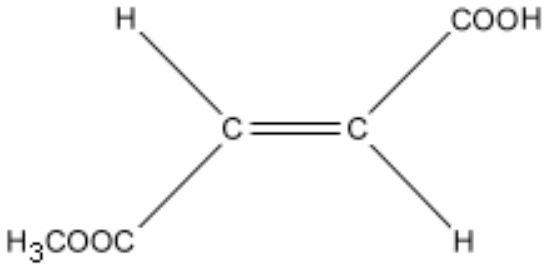
We placed the mixture into an ultrasonic bath for a minimum of 15 min. The temperature rose to 35°C. The solution remained optically stable. After cooling and filling the volumetric flask to the mark with distilled water, the final concentration of DMF was 2 mg/ml.

Perfusion solution of DMF

For control experiments in the isolated perfused rat heart, we added DMF to the Krebs-Henseleit perfusion buffer containing 0.004% DMSO.

3.2.6.2 Methylhydrogen fumarate

Chemical structure:



Chemical formula: $C_5H_6O_4$

Molecular weight: 130 g/mol

Manufacturer: Fumapharm, Muri, Switzerland

Stability: solid: stable, solution: should prepared daily

Function: Metabolite of DMF

Perfusion solution of DMF

For control experiments in the isolated perfused rat heart, we added methylhydrogen fumarate (MHF) to the Krebs- Henseleit perfusion buffer.

3.2.6.3 Intravenous formulation of vehicle

Into a 10 ml volumetric flask we pipetted 200 µl DMSO and filled the flask to the mark with distilled water.

3.2.6.4 Phthalocyanine blue dye

We diluted phthalocyanine blue dye (W-4123, Engelhard, Iselin, USA) with distilled water 1:1 to obtain the ideal consistency determined in pilot experiments. A solution of low viscosity did not stop the heartbeat whereas a solution of high viscosity resulted in a non-uniform staining.

3.2.6.5 2,3,5-triphenyltetrazolium chloride (TTC)

We dissolved TTC in warm water. The 1% solution was of light yellowish color.

3.2.7 Evaluation and statistical analysis

3.2.7.1 In vivo experiments

We expressed infarct size as mean ± SD after confirmation of normal distribution of the data by Shapiro-Wilk test. Comparisons of infarct size between groups were performed using ANOVA followed by Student-Newman-Keuls test with a 0.05 two-sided significance level.

To avoid type II errors (missing significant differences when the sample size is too small) we determined the required sample size for the present study using an approximation for an equal to 0.05 and power to 0.90 (Dawson-Saunders & Trapp 1990). Accordingly, a sample size of 17 rats in each group had 90% power to detect differences in mean infarct size of 20% (the difference between the mean infarct size in control animals and in DMF-treated animals or in preconditioned animals; infarct size given as percentage of the ischemic risk area) assuming that the common standard deviation (SD) is 17% (taken from previous data recorded in identical experimental conditions) (Barbosa et al 1996) using ANOVA with a 0.05 two-sided significance level. Considering 5 pilot experiments and a 15-20% mortality of this model (mostly due to sustained ventricular tachyarrhythmias), we estimated 65 rats to perform this study.

3.2.7.2 Control experiments in the isolated perfused rat heart

We expressed numerical variables as mean ± SD, and compared among groups by ANOVA. Statistical computations were done using Prism software (GraphPad, San Diego, CA, USA; version 3.0a).

4 Results

4.1 Losartan against reperfusion arrhythmias

4.1.1 Aortic banding

To determine the ideal inner diameter of the clip, we performed pilot experiments using different inner diameters. An inner diameter of 0.3 mm (n = 8) provoked a paralyzation of the posterior extremeties and subsequent death of the rats. A dimension of 0.4 mm (n = 28) provoked hypertrophied hearts after a certain time, whereas a diameter of 0.45 mm (n = 8) did not provoke hypertrophy. Therefore we decided to use an inner diameter of 0.4 mm for our experiments. We confirmed left ventricular hypertrophy by magnetic resonance spectroscopy (Fig. 19). Imaging data revealed clear signs of LV hypertrophy in rats after aortic banding including increased LV mass and LV wall thickness (Table 1). However, ejection fraction was similar in both groups indicating compensated LV hypertrophy in banded rats. The wet heart weight/body weight ratio (determined post mortem), was significantly increased in the banded group and therefore confirmed the findings from the magnetic resonance spectroscopy (Fig. 20).

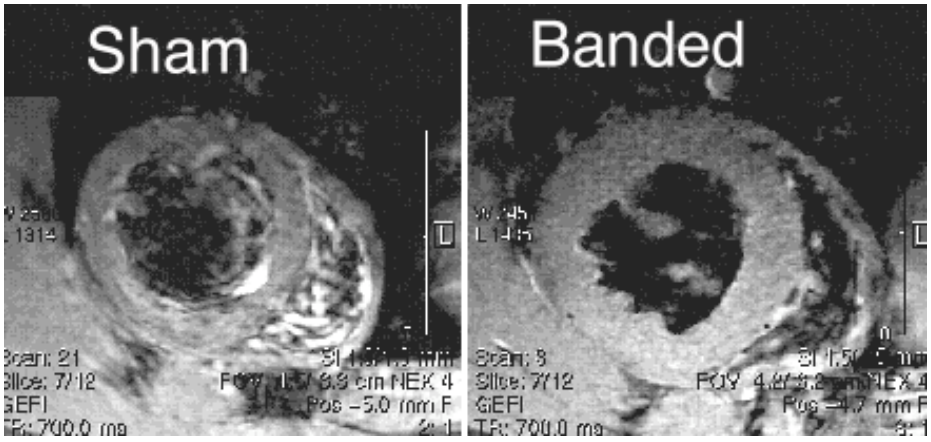


Figure 19: Magnetic resonance images of rat hearts in vivo of a sham (left) and of a banded animal (right) 70 days after surgery.

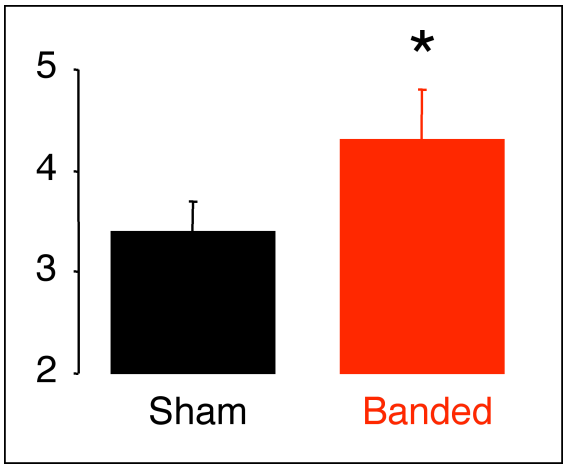


Figure 20: Ratio wet heart weight/body weight (g/kg) 70 days after aortic banding surgery (Mean ± SD, n = 28 and 18 hearts, respectively).

Table 1: Characterization of rat model of left ventricular hypertrophy

	Aorta banded	Sham-operated
Body weight (g)	477 ± 41	488 ± 51
Heart wet weight (g)	2.04 ± 0.32	1.67 ± 0.21 *
Ratio wet heart weight/body weight (g/kg)	4.3 ± 0.5	3.4 ± 0.3 *
LV mass (g)	0.98 ± 0.03	0.70 ± 0.11 *
LV end-diastolic volume (μl)	753 ± 21	659 ± 76
LV end-systolic volume (μl)	316 ± 88	209 ± 35
LV stroke volume (μl)	338 ± 66	450 ± 42
LV wall thickness (mm)	2.4 ± 0.1	1.7 ± 0.3 *
Ejection fraction	0.58 ± 0.10	0.68 ± 0.02

Values are mean ± SD 70 days after surgery (n=28 and 18 hearts, respectively, for weights and n=3 hearts each for magnetic resonance data). Left ventricular (LV) wall thickness was measured in diastole. LV mass was calculated from the end-diastolic myocardial volume multiplied by the myocardial specific gravity (1.05 g/cm^3). The ejection fraction was calculated from the ratio of stroke volume / end-diastolic volume. The stroke volume was calculated as end-diastolic minus end-systolic volumes. *P < 0.05 vs. sham-operated rats.

4.1.2 Dose response curves

In dose-response curves of losartan and enalaprilat on $MAPD_{90\%}$ in non-hypertrophied hearts, neither drug significantly affected $MAPD_{90\%}$ at a constant heart rate of 240 beats per minute (Fig. 21). Similarly, $MAPD_{90\%}$ of control hearts (no drug treatment, n = 3) remained unaltered and stable throughout the duration of the experiment. In contrast, amiodarone (10^{-5} M), which was used as positive control, significantly prolonged the $MAPD_{90\%}$ from 41.9 ms to 52.2 ms (n = 3).

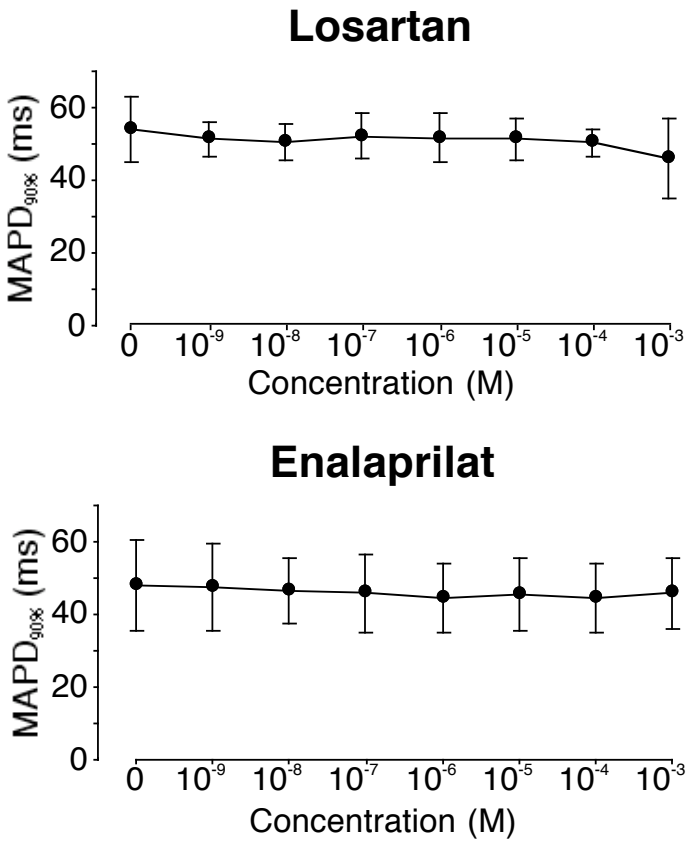


Figure 21: Dose-response curve of losartan and of enalaprilat on monophasic action potential duration at 90% repolarisation ($MAPD_{90\%}$) recorded at a constant heart rate of 240 beats per min in non-hypertrophied rat hearts. Values are mean ± SD of 4 hearts per drug. Note that neither drug significantly affected $MAPD_{90\%}$.

Because none of the tested concentrations of losartan or of enalaprilat affected $MAPD_{90\%}$, we carried out further experiments using concentrations that have previously been shown to be cardioprotective under similar conditions (Grover et al 1991; Zhu et al 1999). Therefore, we used 1 µM losartan (Zhu et al 1999) and 10 µM enalaprilat (Grover et al

1991) for VF threshold and ischemia/reperfusion experiments. These concentrations correspond to the plasma concentrations achieved in humans after oral administration of the drug in a therapeutic dosage (Yasar et al 2002; Najib et al 2003).

4.1.3 VF threshold

Similar to their effects on $MAPD_{90\%}$, neither 1 µM losartan nor 10 µM enalaprilat significantly affected VF threshold (Fig. 22). In contrast, 3 µM lidocaine, which was used as positive control, increased VF threshold to >10V. As a prerequirement for valid and reproducible measurements, we could not detect any differences in VF threshold among groups before drug treatment.

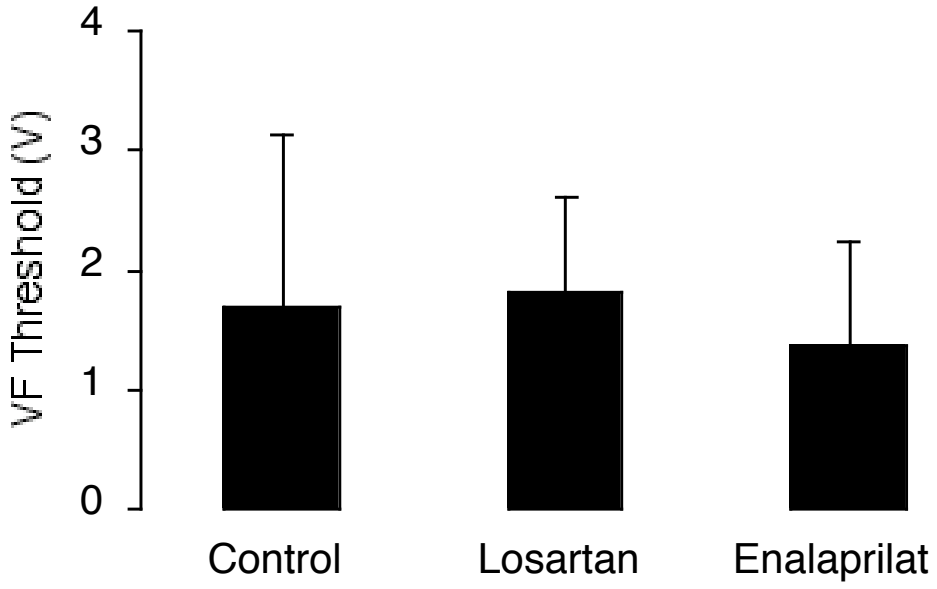


Figure 22: Effect of losartan (1 µM) and of enalaprilat (10 µM) on ventricular fibrillation (VF) threshold in non-hypertrophied rat hearts. Control hearts were perfused with the vehicle only. Values are mean ± SD of 5 to 6 hearts per group. Note that neither drug sigificanty affected VF threshold.

4.1.4 Hemodynamic variables

Hemodynamic variables were similar in all groups throughout the experiments (Table 2). Specifically at baseline, we could not detect any significant differences of coronary flow and LV developed pressure among the groups. Inducing low-flow ischemia by reducing the perfusion pressure led to a consistent reduction of coronary flow and LV developed pressure from 23.2±4.2 ml min^{-1} to 2.5±1.2 ml min^{-1} (=11% residual coronary flow) and from 84.5±15.5 mm Hg to 13.1±5.7 mm Hg, respectively. Importantly, throughout low-flow ischemia, coronary flow and LV developed pressure values did not significantly differ among the groups. Finally, at the end of the reperfusion, coronary flow and LV developed pressure were similar in all groups, recovering to almost baseline levels.

Table 2: Hemodynamic variables

	Hypertrophied hearts			
	Control	**Losartan**	**Enalaprilat**	**Sham-operated**
Coronary flow (ml min^{-1})				
Baseline	21.3 ± 2.3	21.1 ± 4.4	24.8 ± 4.2	24.4 ± 4.3
Low-flow ischemia	3.1 ± 1.4	2.7 ± 1.2	2.9 ± 1.2	2.1 ± 1.1
End reperfusion	18.6 ± 2.9	16.0 ± 5.0	18.6 ± 5.6	14.6 ± 3.2
LV developed pressure (mm Hg)				
Baseline	90.3 ± 29.4	80.6 ± 12.2	80.2 ± 9.4	85.2 ± 5.5
Low-flow ischemia	14.6 ± 6.3	15.7 ± 6.3	12.0 ± 5.5	11.5 ± 5.1
End reperfusion	85.9 ± 28.8	79.9 ± 10.8	69.2 ± 21.4	74.5 ± 17.8

Low-flow ischemia values are averaged over the entire 60 min-period. Values are mean ± SD of 10, 9, 9, and 18 hearts. Neither coronary flow nor left ventricular (LV) developed pressure differed among groups.

4.1.5 Analysis of ventricular arrhythmias

In contrast to their effects on $MAPD_{90\%}$ and on VF threshold, losartan and enalaprilat had differential effects on ventricular tachyarrhythmias induced by low-flow ischemia and reperfusion in hypertrophied hearts (Table 3 and Fig. 23; characterization of ventricular hypertrophy in Table 1). Specifically, during low-flow ischemia, neither drug significantly affected the incidence or median duration of VT or of VF. During reperfusion, however, 1 µM losartan significantly reduced the median duration of tachyarrhythmias (from 51 s to 0 s) to a range observed in non-hypertrophied hearts of sham-operated rats (Fig. 23). Enalaprilat appeared to reduce the duration of tachyarrhythmias during reperfusion too (4 s), but this reduction was not statistically significant (p=0.24). Similarly, the duration of ventricular tachyarrhythmias appeared longer in hypertrophied hearts (51 s) than in non-hypertrophied hearts from sham-operated rats (4 s), but again this difference was not statistically significant (p=0.60).

Table 3: Incidence of VT and of VF during low-flow ischemia and reperfusion

	Hypertrophied hearts			
	Control	**Losartan (1µM)**	**Enalaprilat (10 µM)**	**Sham-operated**
Ischemia				
VT (%)	80	67	100	89
VF (%)	90	56	89	78
Reperfusion				
VT (%)	70	33	67	67
VF (%)	40	22	11	39
n (hearts)	10	9	9	18

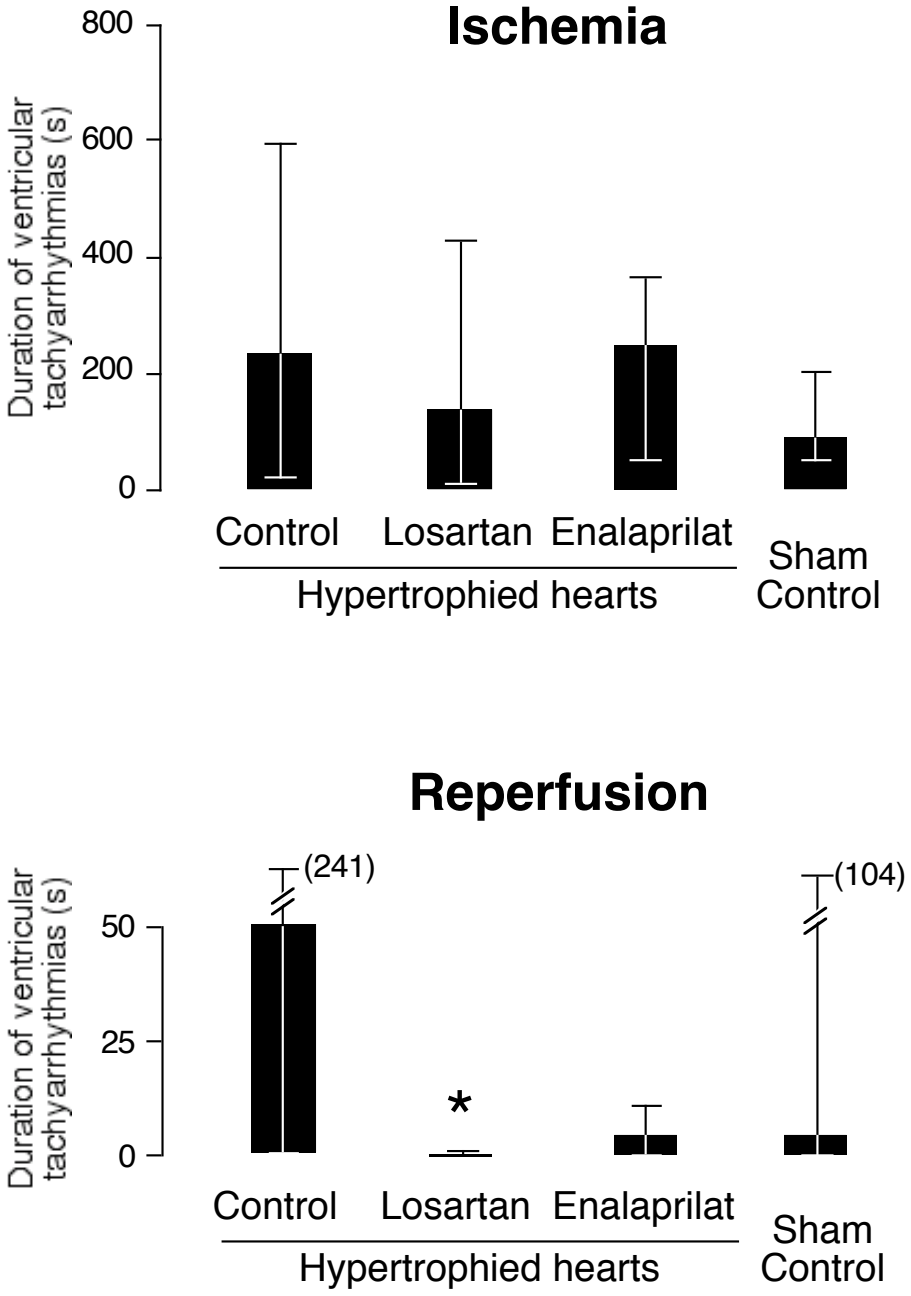


Figure 23: Effect of losartan (1 μM) and of enalaprilat (10 μM) on the duration of ventricular tachyarrhythmias (sum of VT and VF duration) during low-flow ischemia (60 min) and reperfusion (60 min) in hypertrophied rat hearts. Control hypertrophied hearts and sham-operated (non-hypertrophied) hearts were perfused with the vehicle only. The data are shown as median and quartiles of 9, 9, 10, and 18 hearts. *$p < 0.05$ vs. control. Note that losartan significantly reduced the duration of ventricular tachyarrhythmias during reperfusion.

4.2 Dimethyl fumarate against myocardial infarction

4.2.1 Osmolarity of DMF solution

As it is important that the osmolarity of an intravenous formulation corresponds to the osmolarity of blood (290 mosmol/kg) we determined the osmolarity of our solutions. DMF dissolved in distilled water (307 mosmol/kg) and the vehicle prepared with distilled water (291 mosmol/kg) matched the required value. Whereas DMF dissolved in saline (590 mosmol/kg) and the vehicle prepared with saline (576 mosmol/kg) showed a much to high osmolarity. We therefore decided to use distilled water to prepare the intravenous solutions.

4.2.2 Variables

4.2.2.1 Infarct size

Myocardial infarct size was significantly smaller in rats that had received DMF or ischemic preconditioning than in control rats (independent whether infarct size was expressed as percentage of risk area or of left ventricular mass). As required for the infarct data to be valid, the area at risk was similar in all groups (Table 4).

Table 4: Infarct mass in relation to risk mass and LV mass as well as risk mass in relation to LV mass.

	Control	DMF	Preconditioned
Infarct mass / risk mass (%)	28.2 ± 6.2	20.7 ± 9.7*	14.6 ± 9.8*
Infarct mass / LV mass (%)	13.0 ± 4.7	9.1 ± 4.4*	6.7 ± 4.3*
Risk mass / LV mass (%)	44.7 ± 10.1	44.3 ± 5.8	48.6 ± 10.0
Sample size (rats)	15	17	17

Mean ± SD; LV, left ventricular; *P<0.05 vs. control

4.2.2.2 Electrocardiogram, arrhythmias, heart rate, and QT intervall

We could obtain a standard lead II electrocardiogram of good quality in all experiments at baseline. However, during the experiment, we frequently recorded severe electrocardiographic artifacts due to electric interferences of the cautery system (particularly during preparation of the parallel animal) as well as due to manipulations of the electrodes during snare occlusion and release. These artifacts rendered the electrocardiogram unreliable and potentially flawed. Therefore, we did not analyze ventricular arrhythmias during ischemia and reperfusion.

The heart rate of the rats before, during, and after ischemia did not reveal any differences among control, DMF-treated and preconditioned rats (Figure 24).

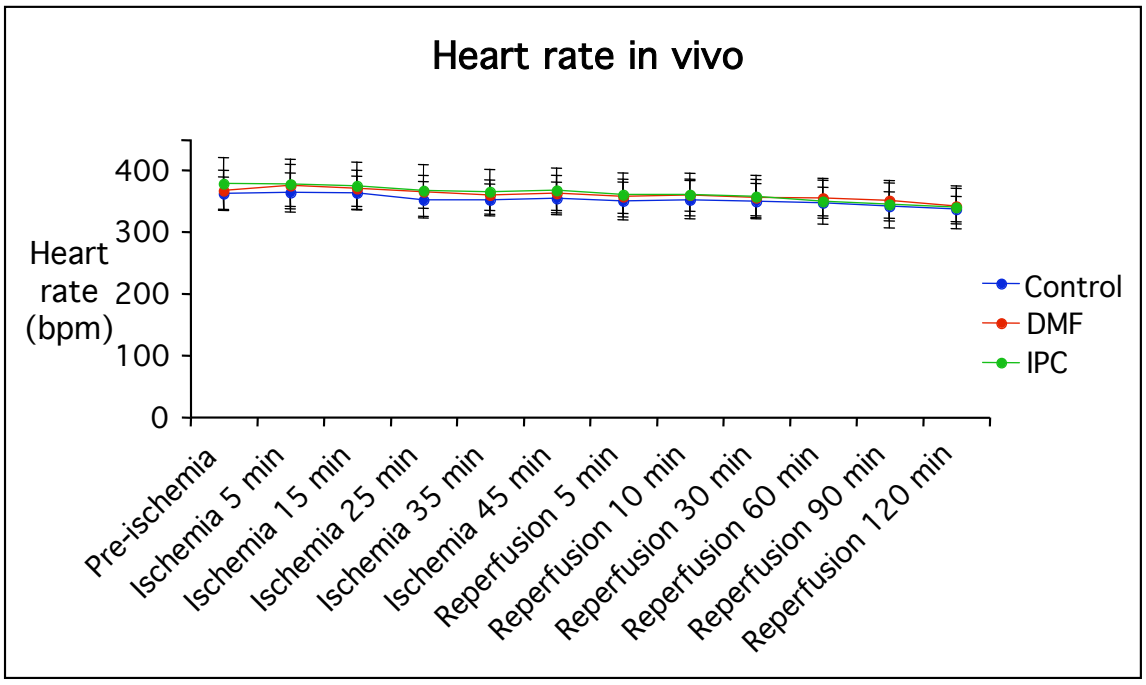


Figure 24: Time-course of heart rate. All groups showed similar heart rate throughout the experimental period (mean ± SD).

Similarly, neither QT time nor QTc-Bazett or QTc-Framingham differed between DMF-treated and untreated animals (Table 5). For this analysis only 15 experiments were analyzed. In all other experiments, the quality of the ECG did not allow realiable QT interval analysis. Therefore we pooled DMF-untreated animals (i.e. control and preconditioned rats, n=3 and 4, respectively) and compared them against DMF-treated animals (n=8).

Table 5: QT and QTc intervals in DMF-treated (n = 8) and DMF-untreated (control and preconditioned, n = 3 and 4, respectively) rats. p = 0.33, p = 0.59, p = 0.37, and p = 0.36, respectively.

	DMF (n=8)	Untreated (n=7)
Heart rate (bpm)	377 ± 29.6	392 ± 31.3
Uncorrected QT interval (seconds)	0.064 ± 0.012	0.067 ± 0.009
QTc-Bazett (seconds)	0.160 ± 0.027	0.172 ± 0.023
QTc-Framingham (seconds)	0.193 ± 0.011	0.198 ± 0.009

Values are mean ± SD of DMF-treated (n = 8) and DMF-untreated (control and preconditioned, n = 3 and 4, respectively) rats (p = 0.33, p = 0.59, p = 0.37, and p = 0.36, respectively). Note that neither the heart rate nor the QT interval differt significantly.

4.2.3 Control experiments in the isolated perfused heart

4.2.3.1 Dose-response curves

In dose-response curves of DMF and of MHF, neither drug significantly affected heart rate, coronary flow, left ventricular developed pressure, or $MAPD_{90\%}$ in isolated perfused rat hearts (Fig. 25). Similarly, DMSO 0.004% (the vehicle of DMF) did not affect any of these hemodynamic or electrophysiologic variables.

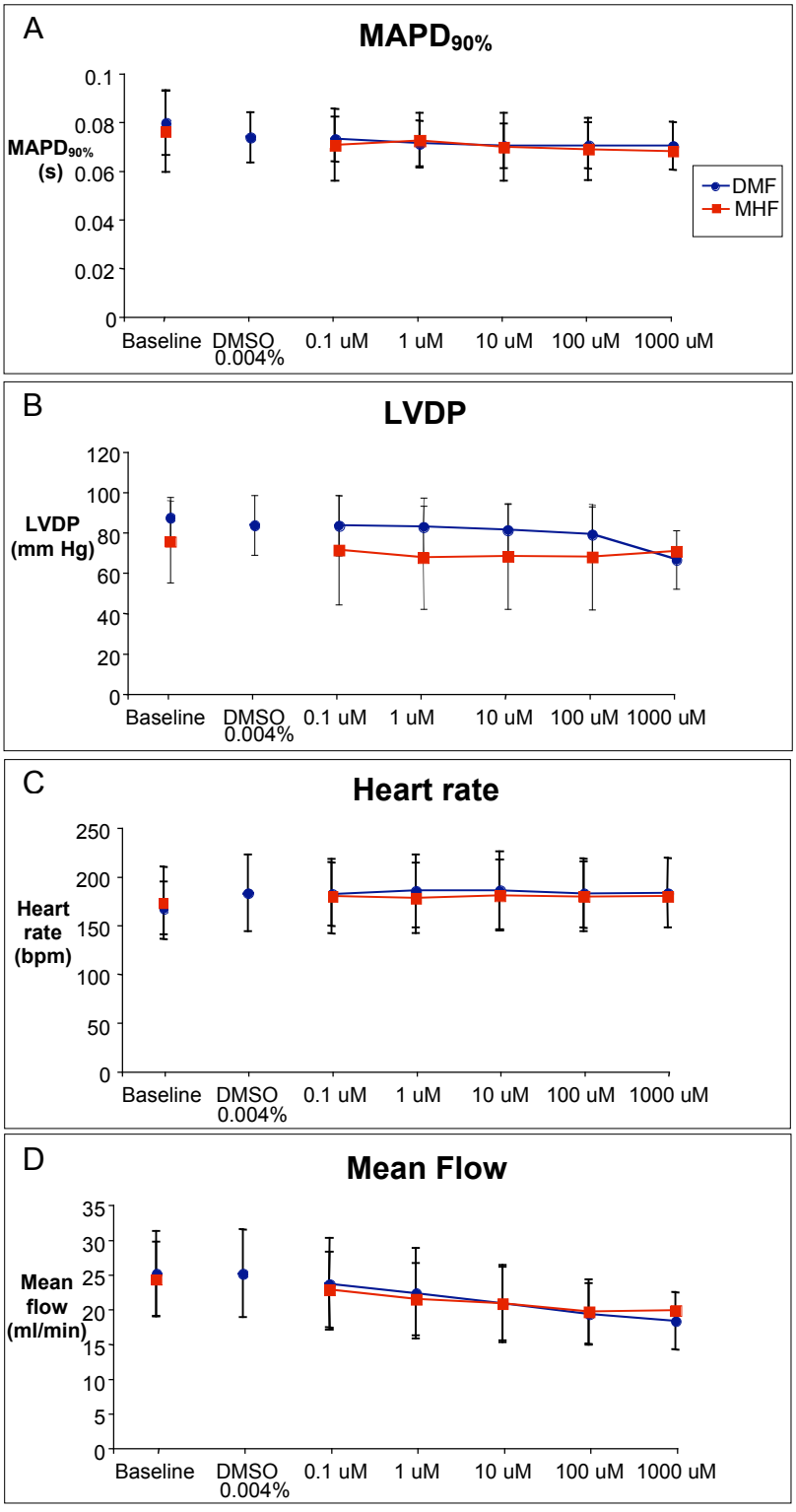


Figure 25: Dose-response curves of DMF (●) and of MHF (■) in isolated perfused rat hearts. Neither drug significantly affected $MAPD_{90\%}$ (A), left ventricular developed pressure (B), heart rate (C), or coronary flow (D).

5 Discussion

5.1 Losartan against reperfusion arrhythmias

The present study in hypertrophied rat hearts demonstrates that neither losartan nor enalaprilat is acutely antiarrhythmic during low-flow ischemia. During reperfusion, however, losartan but not enalaprilat exerts acute antiarrhythmic effects. Specifically, during low-flow ischemia, neither drug reduced the median duration of ventricular tachyarrhythmias. However, during reperfusion, losartan but not enalaprilat reduced the median duration of ventricular tachyarrhythmias indicating acute antiarrhythmic effects (in concentrations comparable to those in human plasma after oral administration of the drug in a therapeutic dosage (Yasar et al 2002; Najib et al 2003)).

These different electrophysiological and antiarrhythmic effects of losartan and enalaprilat partially agree with and extend previous reports about acute antiarrhythmic effects of AT_1 blockers and ACE inhibitors. Similar to our findings, losartan (10 μM) only exerted electrophysiologic and antiarrhythmic effects in early reperfusion but not under normoxic conditions in guinea pig ventricles (Thomas et al 1996). Similarly, in isolated rat hearts an AT_1 blocker reduced the median duration of VF during reperfusion (Fleetwood et al 1991). Moreover, during reperfusion after left descending coronary artery occlusion, losartan (50 $\mu g\ kg^{-1}\ min^{-1}$) beneficially affected both the VF threshold and the incidence of ventricular tachyarrhythmias in dogs (Matsuo et al 1997). Finally, losartan (2 $mg\ kg^{-1}$) reduced the incidence of VT (but not of VF) during reperfusion in rats *in vivo* (Ozer et al 2002). These reports, as well as our findings, support the notion that angiotensin II is a mediator of reperfusion-induced tachyarrhythmias (Fleetwood et al 1991). During low-flow ischemia, however, neither losartan nor enalaprilat significantly affected the incidence or duration of ventricular tachyarrhythmias in our experiments. In this regard, however, it should be considered that this study was not meant and powered to demonstrate differences in the incidence of VF or of VT. Instead, we calculated the median duration of ventricular tachyarrhythmias to obtain a more sensitive measure of potential antiarrhythmic effects. Nevertheless, similar to our results in rats, neither

losartan nor the ACE inhibitor captopril reduced the incidence of ischemia-induced ventricular arrhythmias in dogs (Lynch et al 1999). In contrast, in the setting of myocardial infarction, losartan reduced the incidence of VF in spontaneously hypertensive rats (Lee et al 1997). Furthermore, in contrast to our $MAPD_{90\%}$ data, enalaprilat exerted electrophysiologic effects that caused action potential prolongation in a multisite optical mapping study in isolated guinea pig hearts (Gilat et al 1998). Nevertheless, similar to our findings, this effect was not of sufficient magnitude to suppress the initiation of VF or re-entrant VT.

Interestingly, losartan demonstrated antiarrhythmic effects in hypertrophied hearts during reperfusion without exerting electrophysiologic effects in non-hypertrophied hearts under normoxia. It may thus be speculated that losartan exerts little or no electrophysiologic and antiarrhythmic effects in normal hearts during normoxia. During postischemic reperfusion however, losartan might alter cardiac electrophysiology and therefore be acutely antiarrhythmic. Our study shows for the first time that such an antiarrhythmic effect is present in hypertrophied hearts after low-flow ischemia. This could be of particular relevance to heart failure patients that are at increased risk of life-threatening ventricular tachyarrhythmias induced by low-flow ischemia. The latter, in turn, is a consequence of reduced coronary reserve in hypertrophied hearts. Moreover, losartan showed only antiarrhythmic activity during reperfusion (but not during low-flow ischemia). Therefore, it may be speculated that losartan affects automatic (pacemaker-induced) arrhythmias or triggered arrhythmias.

It is difficult, however, to speculate about electrophysiologic effects of losartan on mechanisms initiating ventricular tachyarrhythmias in our experimental model. This is because global low-flow ischemia presumably generated little flow of injury current and because of ventricular pacing (avoiding asystole during ischemia (Curtis 1998)) that most likely created a site of ectopic automaticity. Still, losartan reduced the duration of ventricular tachyarrhythmias in our experiments and thus, probably affected re-entry mechanisms that maintain ventricular tachyarrhythmias. Such acute antiarrhythmic effects might be mediated by altering angiotensin II-related ventricular electrophysiologic

properties of the ventricles including increasing cardiac refractoriness (de Mello & Crespo 1999), reducing dispersion of action potential duration (De Mello 2001), and prevention of Ca^{2+} overload in cardiomyocytes during reperfusion (Yahiro et al 2003). Additionally or alternatively, acute antiarrhythmic effects of both losartan and enalaprilat may arise from free radical scavenger effects (Birincioglu et al 1997; Donmez et al 2002) and/or the accumulation of bradykinin. The latter possibility does not necessarily favor ACE inhibitors because losartan has been shown to produce bradykinin-dependent cardioprotective effects in rat hearts during ischemia and reperfusion too (Zhu et al 1999). The view that ACE inhibitors and angiotensin do not equally well suppress angiotensin II actions as well as additional effects of losartan on cardiac K^+ currents may explain why losartan but not enalaprilat was acutely antiarrhythmic in our experiments. In this regard, it is important that losartan may exert antiarrhythmic effects independent of AT_1 receptor blockade (Thomas et al 1996) and acutely modified cardiac delayed rectifier K^+ currents (hKv1.5, HERG, and Ks channels; but not Na^+ and Ca^{2+} currents) in guinea pig ventricular myocytes, canine Purkinje fibers or ventricular myocytes (Timmermans et al 1993; Timmermans & Smith 1994; Caballero et al 2000). Consequently, losartan might acutely prolong action potential duration (Caballero et al 2000) and such a prolongation could render the heart less susceptible to re-entrant arrhythmias during early reperfusion when action potential is shortened (Thomas et al 1996).

Unfortunately, we could not record reliable action potentials during ischemia or reperfusion because of varying electrode contacts resulting in action potential artifacts. For this reason, we do not know whether antiarrhythmic effects of losartan were paralleled by electrophysiologic effects during ischemia and reperfusion. Other authors measuring $APD_{90\%}$ in guinea pig ventricles found a shortened $APD_{90\%}$ during simulated ischemia and early reperfusion but no differences between preparations treated with losartan and vehicle (Thomas et al 1996). However, it should be considered that inter-species differences in cellular electrophysiology and action potential morphology limit the comparison of rat hearts to hearts of other species including guinea pigs and human beings. Crucial ionic currents in human beings do not contribute to the repolarisation in rats. Specifically in rats, I_{to} is the most important repolarizing current (Cerbai et al 1994).

In contrast, in human beings, I_{Kr} and I_{Ks}, the two components of the delayed rectifier potassium current, play a dominant role in the repolarisation of the action potential (Fig. 26) (Viswanathan et al 1999; Katz 2001; Rubart & Zipes 2001).

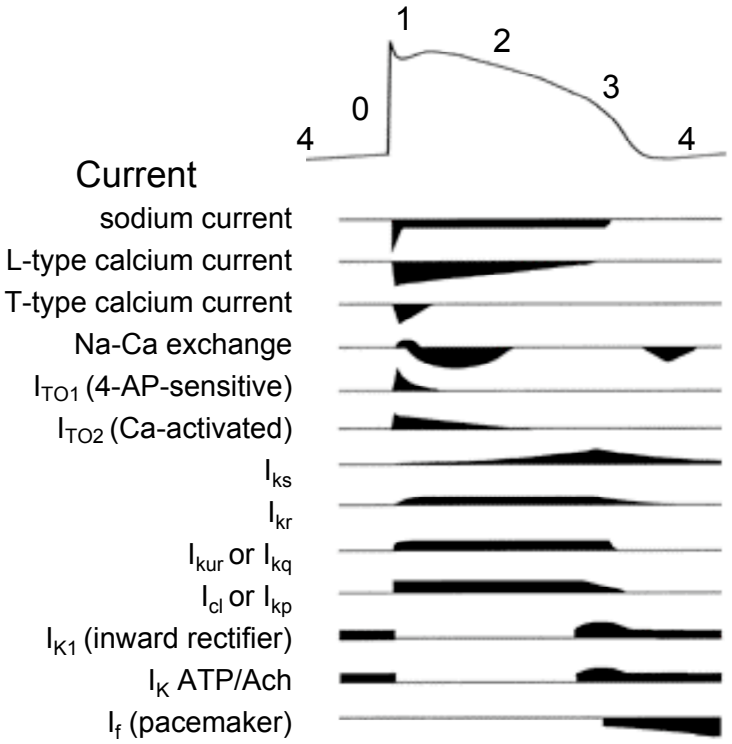


Figure 26: Phases and ionic basis of a purkinje fiber action potential in human beings: Phase 0 (upstroke) corresponds to depolarization, and phase 3 (repolarization) to repolarization in skeletal muscle. Phases 1 (early repolarization) and 2 (plateau) have no clear counterpart in skeletal muscle, while phase 4 (diastole) corresponds to the resting potential. Schematic indication of the time course of depolarizing inward currents (downward) and repolarizing outward currents (upward). Modified from (Katz 2001; Rubart & Zipes 2001).

Nevertheless, in the present study, the rat heart served as a readily available model of LV hypertrophy during ischemia/reperfusion and has previously been used for similar studies (Fleetwood et al 1991; Thomas et al 1996; Lee et al 1997; Matsuo et al 1997; Lynch et al 1999). Furthermore, in the present study, we did not assess effects of chronic administration of drugs as it has been done previously (Zhu et al 2000). It is likely that beneficial effects of AT_1 blockers and ACE inhibitors in patients demonstrating LV hypertrophy and/or heart failure result from chronic effects such as regression of LV hypertrophy (Kohya et al 1995). Taken together, our findings, as well as those of other

authors (Fleetwood et al 1991; Thomas et al 1996; Lee et al 1997; Matsuo et al 1997; Lynch et al 1999) suggest that part of the beneficial effects of losartan may be due to acute actions of this drug. However, the molecular mechanism(s) of these actions and of possible differences to ACE inhibitors remain speculative at present.

5.2 Dimethyl fumarate against myocardial infarction

This study in rats *in vivo* demonstrates that DMF reduces myocardial infarct size after ischemia and reperfusion. Specifically, infarct size of rats that had received DMF (10 mg/kg b.w. as boli i.v. 90 min before ischemia and immediately before ischemia) was significantly smaller than that of control rats that had received only the vehicle (DMSO and water). This is the first description of an antinecrotic effect of DMF in rat hearts.
However, the effect of DMF on myocardial infarct size (-7.5%) was not as pronounced as estimated for calculations of sample size and of statistical power (-20%). This was presumably related to the fact that infarct size in this study was generally smaller than expected (28% in Control rats as opposed to 40-50% in previous studies under similar conditions)(Barbosa et al 1996). This difference was most likely due to isoflurane that was used as an anesthetic agent in the present study but not in previous experiments. Volatile anesthetics, as isoflurane, have cardioprotective properties and can limit infarct size (Cope et al 1997). They are able to mimic cardiac preconditioning by priming the activation of mitochondrial K_{ATP} channels via multiple signaling pathways (Zaugg et al 2002). Still, both DMF and ischemic preconditioning (positive control) reduced myocardial infarct size. This reduction reached statistical significance because not only infarct size but also data variability was lower than in previous studies. The molecular mechanisms for this effect of DMF on myocardial infarct size are presently not clear but may be related to the inhibition of NF-□B. DMF could inhibit the TNF-induced nuclear entry of activated NF-□B in human endothelial cells (Loewe et al 2002). Several other authors could show beneficial effects of NF-□B inhibition in the setting of myocardial infarction. An in vivo transfer of NF-□B decoy oligodeoxynucleotides reduced the extent of myocardial infarction following reperfusion in rat hearts (Morishita et al 1997). These oligonucleotides against NF-□B blocked cytokine and adhesion molecule gene

expression. This led to a decreased infiltration of neutrophiles and reduced inflammatory reactions (Sawa et al 1997). Similarly a targeted NF-κB decoy oligonucleotide could decrease infarct size in pig hearts (Kupatt et al 2002). Inhibition of different NF-κB activating signal pathways alleviated myocardial ischemia/reperfusion injury (Izumi et al 2001; Pye et al 2003). Various pharmacological active agents demonstrated a decrease in infarct size, which the authors constitute with inhibitory effects on NF-κB activation (Squadrito et al 2000; Thourani et al 2000).

DMF is an orally available and well-tolerated drug with few side effects. This may be an advantage compared to other approaches. Gene therapy has still been very controversial (Morishita et al 1997; Sawa et al 1997; Kupatt et al 2002). Other pharmacological agents described to reduce infarct size by inhibition of NF-κB activation are not orally available as heparin (Thourani et al 2000) or have serious side effects as tacrolimus (Squadrito et al 2000).

Due to electrocardiographic artifacts, we could not analyze ventricular arrhythmias. The analysis of heart rate excluded negative chronotropic effects that could have potentially accounted for the cardioprotective effects of DMF in the present study. We could confirm this finding control experiments in isolated beating rat hearts, in which we could show that DMF and MHF do not exert any acute hemodynamic or electrophysiologic effect that could potentially explain antinecrotic effects of DMF in rat hearts. Specifically, neither DMF nor MHF in concentrations ranging from 0.1μM to 1000 μM acutely affected heart rate, coronary flow, left ventricular developed pressure, or $MAPD_{90\%}$ in our experiments. Thus, unlike nitrates, beta-adrenergic receptor blockers, or calcium antagonists that increase myocardial blood flow and/or reduced heart rate, DMF appears to protect hearts against myocardial infarction without acute hemodynamic or electrophysiologic effects. Caution is advised however, when inferring from rat cardiac action potentials because inter-species differences in cellular electrophysiology and action potential morphology limit the comparison of rat hearts to hearts of other species including human beings. In the present study, the rat heart served as readily available model to assess acute hemodynamic and electrophysiologic effects. Still, extrapolations from rat to human

hearts should be done carefully and absence of electrophysiologic effects should be confirmed in a species that is closer to human beings (e.g. guinea pigs, pigs). Nevertheless, at least in rats, antinecrotic effects of DMF are unlikely to be due to decreased heart rate or left ventricular pressure, increased coronary flow, or shortened action potential duration.

Furthermore, the analysis of QTc intervals from the ECG recorded during the in vivo experiments, suggests that at least in rats, DMF did not affect ventricular repolarisation and thus, is unlikely to be proarrhythmic, which is important for a drug to be developed further.

6 Conclusion

6.1 Losartan against reperfusion arrhythmias

The first study demonstrates that neither losartan nor enalaprilat is acutely antiarrhythmic during ischemia. During reperfusion, however, losartan but not enalaprilat exerts acute antiarrhythmic effects. Beneficial effects in heart failure patients might thus not only be due to chronic effects but also due to acute effects of losartan.

6.2 Dimethyl fumarate against myocardial infarction

The second study demonstrated that DMF reduced myocardial infarct size in a rat model of acute ischemic and reperfusion in vivo. The antinecrotic effects of DMF are unlikely to be due to decreased heart rate or left ventricular pressure, increased coronary flow, or shortened action potential duration.

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